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Polymeromics

Structural elucidation of macromolecules via tandem mass spectrometry utilizing various ionization techniques

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kendime ait duygu,düşünce,davranış,yetenek ve becerilerime iyi bakmayı öğrendiğim; hayatımda değisik farkındalıklar geliştirdiğim zorlu doktora sürecimin ürünü olan doktora tezimi kendime armağan ediyorum...

Kendime ☺

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Documentation of authorship

This section contains a list of the individual authors` contributions to the publications reprinted in this thesis.

1) Esra Altuntaş, and Ulrich S. Schubert, "Polymeromics: Mass spectrometry (MS) based strategies in polymer science towards complete sequencing approaches"

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E. Altuntaş:	preparation of the manuscript
U. S. Schubert:	correction of the manuscript, supervision

2) Esra Altuntaş, Kristian Kempe, Anna Crecelius, Richard Hoogenboom, and Ulrich S. Schubert, "Electrospray ionization-mass spectrometric (ESI-MS & MS/MS) analysis of poly(2-oxazoline)s with different side groups"

Macromol. Chem. Phys. **2010**, *211*, 2312-2322.

E. Altuntaş:	characterization of polymers, conceptual development, preparation of the manuscript
K. Kempe:	monomer and polymer synthesis
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R. Hoogenboom:	correction of the manuscript
U. S. Schubert:	correction of the manuscript, supervision

3) Esra Altuntaş, Andreas Winter, Anja Baumgaertel, Renzo M. Paulus, Christoph Ulbricht, Anna C. Crecelius, Nikolaus Risch, and Ulrich S. Schubert, "Determination of the relative ligand binding strengths in heteroleptic Ir^{III} complexes by ESI-Q-TOF tandem mass spectrometry"

J. Mass Spectrom. **2012,** *47*, 37-43.

E. Altuntaş:	characterization of complexes, preparation of the manuscript
A. Winter:	complex synthesis, correction of the manuscript, supervision
A. Baumgärtel:	characterization
R. M. Paulus:	characterization
C. Ulbricht:	ligand synthesis
A. C. Crecelius:	correction of the manuscript
N. Risch:	characterization
U. S. Schubert:	correction of the manuscript, supervision

4) Esra Altuntaş, Katrin Knop, Lutz Tauhardt, Kristian Kempe, Anna C. Crecelius, Michael Jäger, Martin D. Hager, and Ulrich S. Schubert, "Tandem mass spectrometry of poly(ethylene imine)s by electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI)"

J. Mass Spectrom. **2012**, *47,* 105-114.

E. Altuntaş:	characterization of polymers, conceptual development, preparation of the manuscript
K. Knop:	characterization
L. Tauhardt:	polymer synthesis
K. Kempe:	polymer synthesis
A. C. Crecelius:	correction of the manuscript
M. Jäger:	correction of the manuscript
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U. S. Schubert:	correction of the manuscript, supervision

5) Esra Altuntaş, Anja Baumgaertel, Andreas Krieg, Anna C. Crecelius, and Ulrich S. Schubert, "ESI, APCI, and MALDI tandem mass spectrometry of poly(methylacrylate)s: A comparison study for the structural characterization of polymers synthesized via CRP techniques and the software application to analyse MS/MS data"

J. Polym. Sci., Part A: Polym. Chem. **2013**, *51*, 1595-1605 (cover).

E. Altuntaş:	characterization of polymers, conceptual development, preparation of the manuscript
A. Baumgaertel:	characterization
A. Krieg:	synthesis of the polymers
A. C. Crecelius:	correction of the manuscript
U. S. Schubert:	correction of the manuscript, supervision

6) Esra Altuntaş, Christine Weber, and Ulrich S. Schubert, "Detailed characterization of poly(2-oxazoline)s by energy-variable collision-induced dissociation study"

Rapid Commun. Mass Spectrom. **2013**, *27*, 1095–1100.

E. Altuntas:	characterization of polymers, conceptual development, preparation of the manuscript
C. Weber:	synthesis of the polymers, correction of the manuscript
U. S. Schubert:	correction of the manuscript, supervision

7) Esra Altuntaş, Christine Weber, Kristian Kempe, and Ulrich S. Schubert, "Comparison of ESI, APCI and MALDI for the tandem mass analysis of poly(2-ethyl-2-oxazoline)s with different end-groups"

Eur. Polym. J. **2013**, *49*, 2172–2185.

E. Altuntaş:	characterization of polymers, conceptual development, preparation of the manuscript
C. Weber:	correction of the manuscript
K. Kempe:	monomer and polymer synthesis
U. S. Schubert:	correction of the manuscript, supervision

8) Anja Baumgaertel, Esra Altuntaş, Kristian Kempe, Anna Crecelius, and Ulrich S. Schubert, "Characterization of different poly(2-oxazoline) block copolymers by MALDI-TOF MS/MS and ESI-Q-TOF MS/MS"

J. Polym. Sci., Part A: Polym. Chem. **2010**, *48*, 5533-5540.

A. Baumgaertel:	characterization of polymers, preparation of the manuscript
E. Altuntaş:	characterization of polymers, preparation of the manuscript
K. Kempe:	monomer and polymer synthesis
A. Crecelius:	correction of the manuscript
U. S. Schubert:	correction of the manuscript, supervision

9) Anja Baumgaertel, Christine Weber, Nicole Fritz, Grit Festag, Esra Altuntaş, Kristian Kempe, Richard Hoogenboom, and Ulrich S. Schubert, "Characterization of poly(2-oxazoline) homo- and copolymers by liquid chromatography at critical conditions"

J. Chromatogr. A **2011**, *1218*, 8370-8378.

A. Baumgärtel:	characterization of polymers by LCCC, MALDI-MS and 2D-LC, preparation of the manuscript
C. Weber:	synthesis of the polymers, preparation of the manuscript
K. Kempe:	synthesis of the block polymer
N. Fritz, G. Festag:	characterization of the polymers by LCCC, 2D-LC
E. Altuntas:	characterization of the polymers by ESI-Q-TOF MS
R. Hoogenboom:	correction of the manuscript
U. S. Schubert:	correction of the manuscript, supervision

10) Anja Baumgaertel, Esra Altuntaş, and Ulrich S. Schubert, "Recent developments in the detailed characterization of polymers by multidimensional chromatography"

J. Chromatogr. A **2012**, *1240*, 1-20.

A. Baumgaertel:	preparation of the manuscript
E. Altuntaş:	preparation of the manuscript
U. S. Schubert:	correction of the manuscript, supervision

Jena, _____ _____

Prof. Dr. Ulrich S. Schubert

1. Introduction

Parts of this chapter have been / will be published: P1) E. Altuntaş, U. S. Schubert (Manuscript submitted to *Anal. Chim. Acta*, **2013**). P10) A. Baumgaertel, E. Altuntaş, U. S. Schubert, *J. Chromatogr. A* **2012**, *1240*, 1-20.

A number of polymerization techniques have been developed for the synthesis of well-defined polymers such as controlled radical polymerizations (CRP)[1, 2] (*i.e.* reversible addition fragmentation chain-transfer polymerization (RAFT),[3-6] atom transfer radical polymerization (ATRP)[7-9] and nitroxide mediated polymerization (NMP)[10, 11]) and living ionic polymerizations (*i.e.* living anionic polymerization and living cationic ring-opening polymerization (CROP)[12-14]). The general aim of these polymerization techniques is to achieve complete control over the structure and molar mass of the polymer to produce easily accessible tailor-made polymers for a variety of applications. The synthetic efforts during the last decades have led to significant advancements of the synthetic pathways. However, the precise characterization of the obtained polymers is still a great challenge in this field. Therefore, nowadays, the accurate characterization of synthetic polymers is one of the most important targets of modern polymer science. There are several powerful characterization tools such as size-exclusion chromatography (SEC) as well as nuclear magnetic resonance spectroscopy (NMR) for the characterization of synthetic polymers and they have served the polymer community for a long time. Nevertheless, these traditional methods have certain limitations and drawbacks. For that reason, current research in polymer science and engineering has to move from these traditional methodologies to sophisticated analytical strategies in which mass spectrometry (MS) techniques play a crucial role.

The characterization of natural and synthetic polymers represents certainly an important part of polymer science, because the main goal is to advance the performance of polymeric material which is associated to its physical properties. The molar mass is one of these properties which affect the resulting characteristics of polymeric material. Obtaining accurate information regarding the molar masses, polydispersity index (PDI) values, and end-group structures in synthetic polymers is an analytical issue which can be addressed successfully by MS techniques. The application of mass spectrometry to analyze synthetic polymers is a fast and reliable method, and suitable for the study of many synthetic polymers with minimal sample consumption.[15-18] A variety of MS techniques have been utilized for the characterization of different macromolecules in recent years owing to the development of soft ionization techniques such as electrospray ionization mass spectrometry (ESI-MS),[19] matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)[20, 21] and atmospheric pressure chemical ionization mass spectrometry (APCI-MS).[22] Even though these methods involve different ion formation processes, they generally allow the ionization of different macromolecules with little or no fragmentation. As a consequence, information about molar mass distributions and end-group functionalities can be obtained. On the other hand, molar mass information alone is not sufficient to derive detailed information of the macromolecular structure of polymers. Supplementary structural information can be obtained using tandem mass spectrometry

(MS/MS or MS^n) analysis to identify individual end-groups, to differentiate isobaric and isomeric species as well as to analyze the macromolecular architectures of polymers in detail.[23, 24] In MS^n, collision-induced dissociation (CID) experiments can be performed upon precursor ions of interest in order to gain further structural information from the resulting fragmentation patterns and to understand the fragmentation mechanism of various polymers. Subsequent to these investigations, a characteristic fragmentation pattern can be defined for a certain type of synthetic polymer and can be used as a reference to characterize unidentified polymers within the same class. On the other hand, fragmentation pathways of the same class of synthetic polymers may be different from the well-known dissociation behavior in case of polymers with fragile end-groups, for instance, the polymers obtained from different CRP techniques. Initiating (α) and/or terminating (ω) end-groups can definitely influence chain-end and in-chain bond cleavages of synthetic polymers. As a consequence, it is extremely important to scrutinize the same polymer class with different end-groups to discover the differences in fragmentation pathways. Moreover, the application of various ionization techniques (*e.g.* ESI-MS, MALDI-MS and APCI-MS) is also important in order to achieve the best analysis conditions.

Additionally, it is required to analyze synthetic polymers in different excitation conditions and as a function of their composition in order to gain a good understanding of their fragmentation behavior. This prompts the need for the analysis of polymers *via* energy-dependent collision induced dissociation (CID) experiments to investigate the dependency of the fragmentation patterns as a function of collision energy. Survival yield (SY) analysis is used in mass spectrometry as a tool for evaluating precursor ion stability and internal energy. Obtaining SY curves from the energy-dependent MS^n data provides a useful way of presenting all information of the CID processes undergone by a specific type of polymer ion. Characteristic collision energy (CCE or CE_{50}) values can be easily determined from the SY curves and these values could discriminate between different compounds (even structural and positional isomers).[25, 26] The SY method reveals insights about the energy requirements of the fragmentation pathways of polymers.[27] Therefore, it is a promising way for identification and discrimination of polymers with different functionalities or backbones. This kind of MS^n studies is required to obtain a complete picture of the fragmentation mechanisms of polymers.

As mentioned previously, MS^n offer accurate structural information from intricate macromolecular structures; however, it produces vast amount of data to interpret. In *-omics* sciences, software tools to interpret the obtained data has developed satisfyingly (*e.g.* in proteomics), because it is not possible to handle the amount of data acquired via (tandem) MS studies on the biological samples manually. It can be expected that special software solutions will improve the interpretation of the MS^n output from the investigations of synthetic polymers as well. Eventually, the MS^n field will also open up for polymer scientists who are not MS-specialists. On the way to "Polymeromics" [28, 29] (Figure 1), tailor-made software tools will have an immense importance in the sequential analysis of polymers and they will help to characterize synthetic polymers by MS^n approaches and, thereby, enabling a frequent application as in the fields of proteomics, metabolomics, genomics, foodomics and glycomics. [30-36]

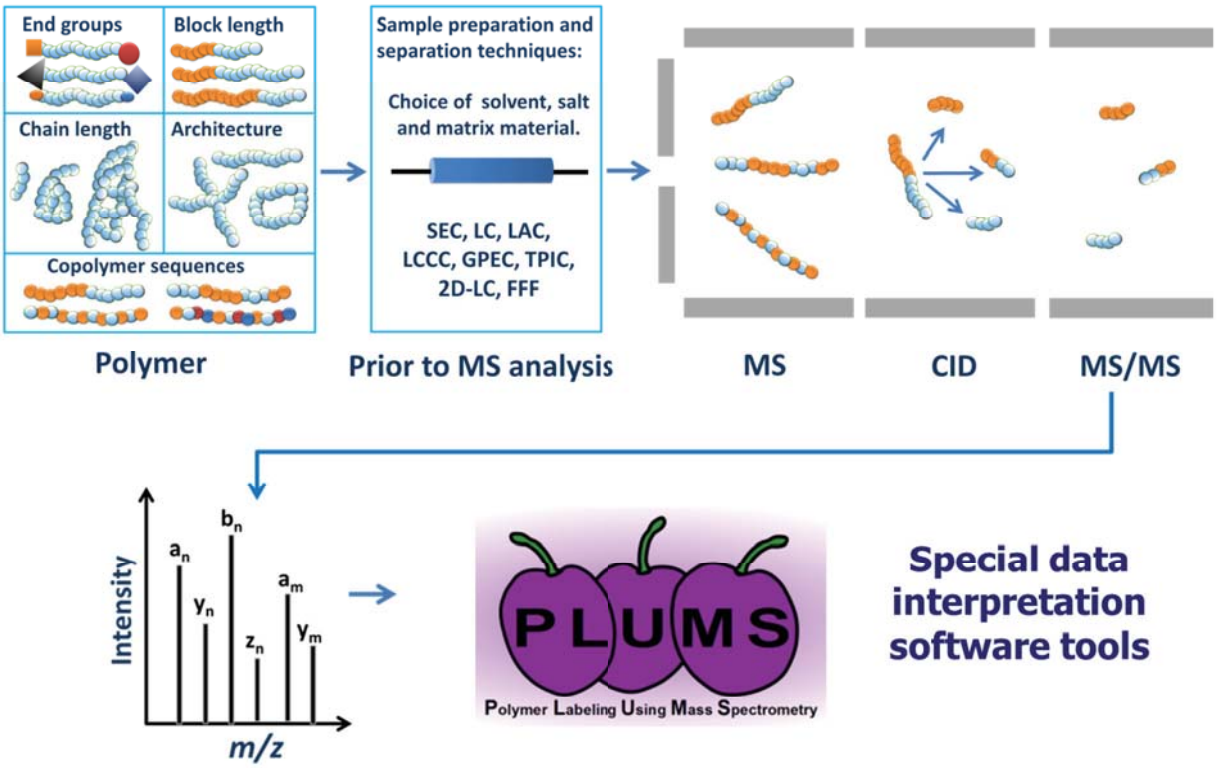


Figure 1.1 "Polymeromics": General outline for the characterization of polymers via mass spectrometry-based strategies. Polymer samples can be fractionated by various separation techniques prior to MS analysis (not obligatory). Mass to charge (*m/z*) ratios are measured from polymer ions that pass the collision cell without fragmentation in the mass spectrometer (MS). Specific ions are selected for collision induced dissociation (CID) experiments and the resulting fragment ions are measured in the second mass analyzer in tandem mass spectrometry (MS/MS). Special data interpretation tools can be optionally utilized for faster evaluation of the data.

The purpose of this thesis is to demonstrate the utilization of advanced (tandem) mass spectrometry techniques in order to characterize synthetic polymers in a comprehensive manner. In Chapter 2, a variety of synthetic polymer classes will be examined in detail on the way to their structural depiction via single-dimensional MS in order to gain information about their molar mass distributions, polydispersity index (PDI) values, monomer units, macromolecular architectures, side chain substituents and end-group functionalities. An assessment of different ionization techniques such as ESI-MS, MALDI-MS and APCI-MS will be scrutinized in Chapter 3 in order to gain a perspective on various ionization techniques for the analysis of polymers. The prospect of analyzing various macromolecules by tandem MS (MS^n) will be discussed in Chapter 4. A line of investigations have been followed in order to achieve the fragmentation behavior of several synthetic polymer classes such as various poly(2-oxazoline)s (POx), poly(ethylene imine)s (PEI), and poly(methyl acrylate)s (PMA). The effect of side-groups and end-groups on the fragmentation pathways will be evaluated in detail to draw conclusions. Subsequent to exploring the potential of the MS^n methods with respect to their capabilities of investigating end-group functionalities, side-chain substituents and macromolecular architectures, MS^n will be further utilized to observe the fragmentation behavior of polymers and supramolecular substances in energy dependent CID experiments. An introduction to this field will be provided along with the obtained results in Chapter 5. MS-based techniques can be

coupled with certain separation methods such as liquid chromatography (LC) in order to face the complexity of synthetic polymers. These techniques offer a versatile way for the quantitative and qualitative analysis of synthetic polymers due to their sensitivity and specificity. Hyphenation of LC and MS techniques for polymer characterization will be shown for the case of poly(2-ethyl-2-oxazoline)s (PEtOx) in Chapter 6. An important approach for the evaluation of the MS^n data by a special interpretation software will be described in Chapter 7. The field of "Polymeromics" will be introduced in detail with selected examples.

In the future, the knowledge gained during this thesis can be further utilized to analyze more complex polymers via (tandem) MS. The fragmentation pathways proposed will be available for all polymer scientists around the world for further research. On the origin of the results offered, the software studies will be enlarged to cover more complex systems like block, graft and random copolymers.

2. Mass spectrometry of synthetic polymers

Parts of this chapter have been published: P2) E. Altuntaş, K. Kempe, A. Crecelius, R. Hoogenboom, U. S. Schubert, *Macromol. Chem. Phys.* **2010**, *211*, 2312-2322. P4) E. Altuntaş, K. Knop, L. Tauhardt, K. Kempe, A. C. Crecelius, M. Jäger, M. D. Hager, U. S. Schubert, *J. Mass Spectrom.* **2012**, *47,* 105-114. P5) E. Altuntaş, A. Baumgaertel, A. Krieg, A. C. Crecelius, U. S. Schubert, *J. Polym. Sci., Part A: Polym. Chem.* **2013**, *51*, 1595-1605.

The analysis of synthetic polymer samples presents special challenges and limitations because of the complex structure of the materials. The molecular complexity of polymers requires new technologies and approaches to provide detailed structural insights. MS-based techniques, as powerful analytical tools for the identification of the chemical composition, structure and molar mass, can provide solutions for the polymer science community. A number of synthetic polymer classes such as various poly(2-oxazoline)s (POx), poly(ethylene imine)s (PEI), poly(methyl acrylate)s (PMA) and many other polymeric structures have been investigated throughout this thesis to show the advantages of MS-based techniques for the characterization of polymers. The utilization of MS data in polymer analysis is depicted in Figure 2.1 and the simple mass spectra from a PMA homopolymer (**P1**) was used to explain the data analysis process. Information about the molar mass values (M_n, M_w, and PDI) of this polymer can be easily obtained by the help of simple software tools (*i.e.* Polytools, Bruker Daltonics, Bremen, Germany). The molar mass differences between the main distribution's peaks can be assigned to the respective monomer repeating units of the studied polymer (86.04 *m/z* for methyl acrylate in the case of PMA). Initiating (α) and terminating (ω) end-groups can be identified by simply comparing the measured and calculated isotopic patterns (including the correct cationization agent). The detailed characterization of this polymer class will be discussed in the following chapters of the thesis.

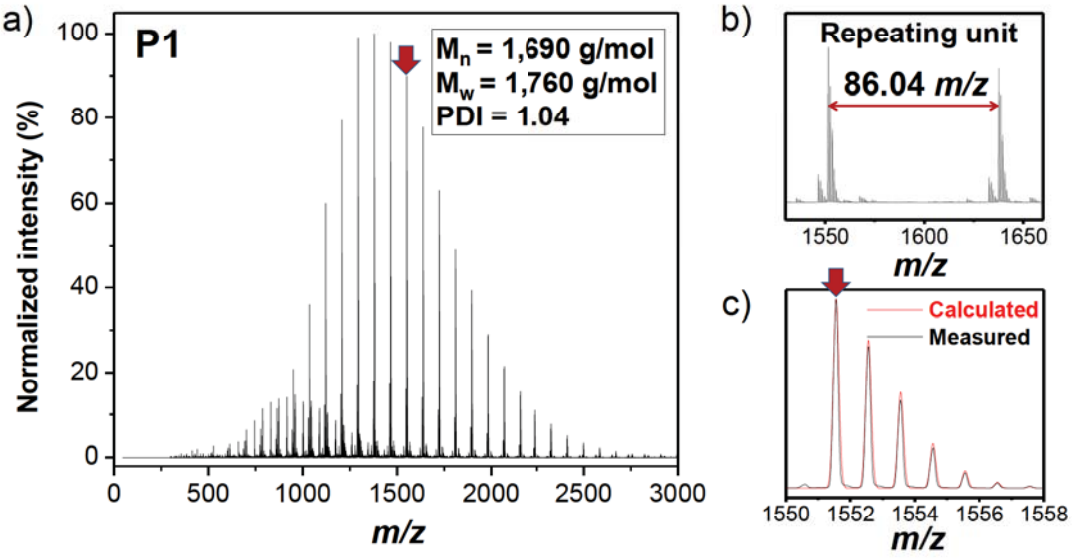


Figure 2.1 a) ESI-Q-TOF mass spectra of PMA homopolymer (**P1**), b) the determination of monomer repeating unit of this polymer, and c) the analysis of end- and side-group functionalities by comparing the measured and calculated isotopic patterns.

In the first part of the thesis, POx homopolymers with different side groups (*i.e.* methyl, ethyl, 1-ethylpentyl, phenyl, *o*-difluoro-phenyl and *p*-*tert*-butylphenyl) were investigated in detail by utilizing the ESI-Q-TOF MS technique. These polymers were synthesized via a microwave-assisted cationic ring-opening polymerization (CROP) and polymer solutions with concentration of 10 µg/mL were subjected to MS analysis.[37]

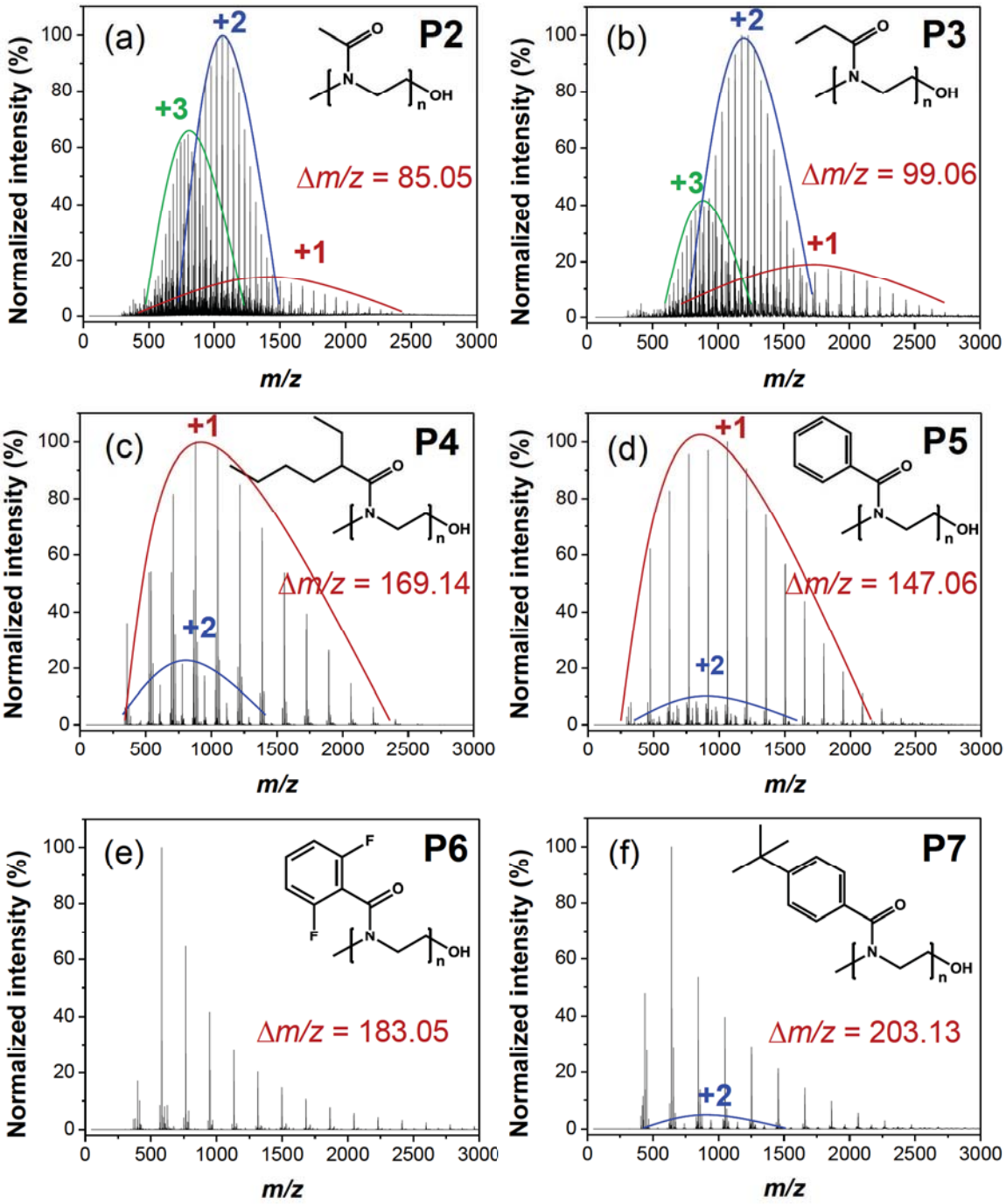


Figure 2.2 ESI-Q-TOF mass spectra of poly(2-oxazoline)s with different side groups: (a) Methyl (MeOx) (**P2**), (b) ethyl (EtOx) (**P3**), (c) ethylpentyl (EPOx) (**P4**), (d) phenyl (PhOx) (**P5**), (e) 2,6-difluorophenyl (ODFOx) (**P6**), and (f) *p*-*tert*-butylphenyl oxazoline (pTBPhOx) (**P7**). Different lines indicate the singly, doubly, and triply charged molar mass distributions (noted as +1, +2, and +3).

As depicted in Figure 2.2, ESI-Q-TOF MS measurements were conducted to provide information about the average molar masses of these POxs and to determine the repeating units as well as the end-groups. The results obtained by the ESI-Q-TOF MS experiments are in a good agreement with the results based on the SEC analysis. For example, the M_n value obtained via ESI-Q-TOF MS for PMeOx (**P2**) is 2,160 g.mol^{-1}, whereas the M_n value obtained via SEC is 2,180 g.mol^{-1}. However, there are some influences in both techniques which might affect the results; therefore, it is difficult to make a clear comparison between these techniques. SEC data can be influenced by the structure of the side groups of different poly(2-oxazoline)s, which may change the hydrodynamic volume of poly(2-oxazoline)s in the SEC solvent or can cause interactions with the column material. Moreover, correct calibration standards are missing for new polymers. For ESI-Q-TOF MS, ionization problems can occur due to the mass discrimination effect when the polymer's PDI value is relatively high (PDI>1.3) and, in particular, for high molar mass polymers. Also, the interpretation of the ESI MS spectra of high molar mass polymers might be complicated due to the overlapping multiply charged species. However, in this study, POx polymers with molar masses around 2,000 to 3,000 g.mol^{-1} were synthesized in order to use these polymers for further characterization with tandem MS. Therefore, it can be assumed that ESI-Q-TOF-MS can provide better results compared to SEC within this molar mass range, because it is not affected by the above discussed problem within the studied molar mass range.

Additionally, ESI-Q-TOF mass spectra show the expected signal spacings for the respective monomers of the investigated POxs (85.05 Δ*m/z* for 2-methyl-2-oxazoline, 99.06 Δ*m/z* for 2-ethyl-2-oxazoline, 169.14 Δ*m/z* for 2-(1-ethylpentyl)-2-oxazoline, 147.06 Δ*m/z* for 2-phenyl-2-oxazoline, 183.05 Δ*m/z* for 2-(2,6-difluorophenyl-2-oxazoline), and 203.13 Δ*m/z* for 2-*p*-*tert*-buthylphenyl-2-oxazoline). The expected polymer chains with methyl as starting group and hydroxyl as end-group were observed as the main distribution in the ESI-Q-TOF mass spectra and the second distribution can be explained by the already known chain-transfer reactions that take place during the polymerization of 2-oxazolines leading to hydrogen initiated chains. In some cases, multiply charged species (doubly (+2) and triply (+3)) were observed. Mass measurement accuracies as ppm error that were calculated from the difference between the calculated and the experimental monoisotopic masses of less than 10 ppm were found for all POx homopolymers.

Similar studies were successfully performed to analyze other synthetic polymer classes by using MS as an efficient approach to reveal the exact mass of the end-groups and monomer units. For instance, PEIs and PMAs with different starting and end-groups were analyzed by ESI-Q-TOF MS to elucidate the macromolecular structures of these polymers in detail.[28, 29]

In another study, a broad library of poly(2-oxazoline) block copolymers were characterized by ESI-Q-TOF MS (Figure 2.3).[38] The ESI-Q-TOF MS spectra were utilized to confirm the copolymer structure by comparing the measured and the theoretical calculated isotopic patterns. The p(EtOx-*b*-EPOx) block copolymer (**P8**) contains two main distributions (methyl and proton initiated copolymers) consisting of different block length of the two monomers. The proton initiated second distribution can be explained by chain transfer reactions during the polymerization process. The varying length of the two blocks is shown by the number of monomer units of the EtOx and the EPOx monomers

(Figure 2.3b). The detailed characterization of different possible structures could be confirmed by tandem MS analysis (which will be discussed in Chapter 4).

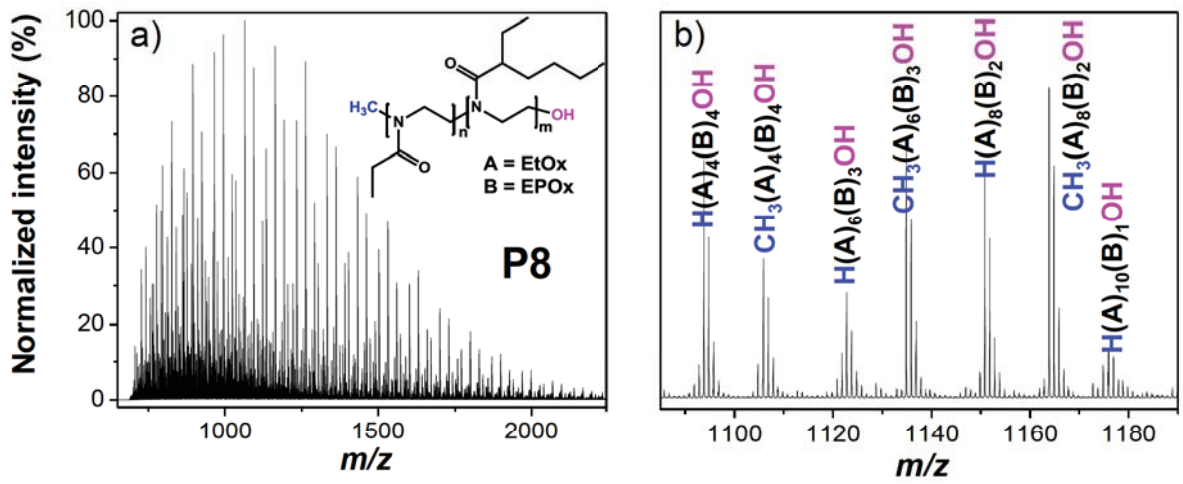


Figure 2.3 (a) ESI-Q-TOF MS spectrum of p(EtOx-*b*-EPOx) copolymer (**P8**) and (b) zoom into the MS spectrum of p(EtOx-*b*-EPOx) (**P8**) (*m/z* 1085 to 1190).

In a different study, a homotelechelic bis-terpyridine containing macroligand (**P9**) synthesized via nitroxide mediated polymerization (NMP) of styrene has been analyzed by ESI-Q-TOF MS. The ESI-Q-TOF MS spectrum revealed two main distributions for this compound corresponding to the doubly and triply charged species, respectively (Figure 2.4). After careful analysis, the main distribution could be assigned to a polystyrene (PS) series, where one of the terpyridine units was cleaved off during the ESI-Q-TOF measurement leaving the central TIPNO moiety still intact. However, the intact telechelic PS bearing both tpy units could be also assigned as a minor distribution. It was not possible to observe any of these species in the MALDI analysis, which proves that these fragile end-groups were cleaved off during the MALDI process. The isotopic pattern obtained from ESI measurement was in good agreement with the calculated one. The calculated pattern for the triply charged species deviated by only *m/z* 0.0004 from the measured value which confirmed the incorporation of both tpy units into the PS. The ESI mass spectrum was deconvoluted to obtain singly charged species; subsequently the M_n value of 8,500 g.mol^{-1} was calculated from the deconvoluted spectrum which is in good correlation with the obtained SEC data for this polymer. [39]

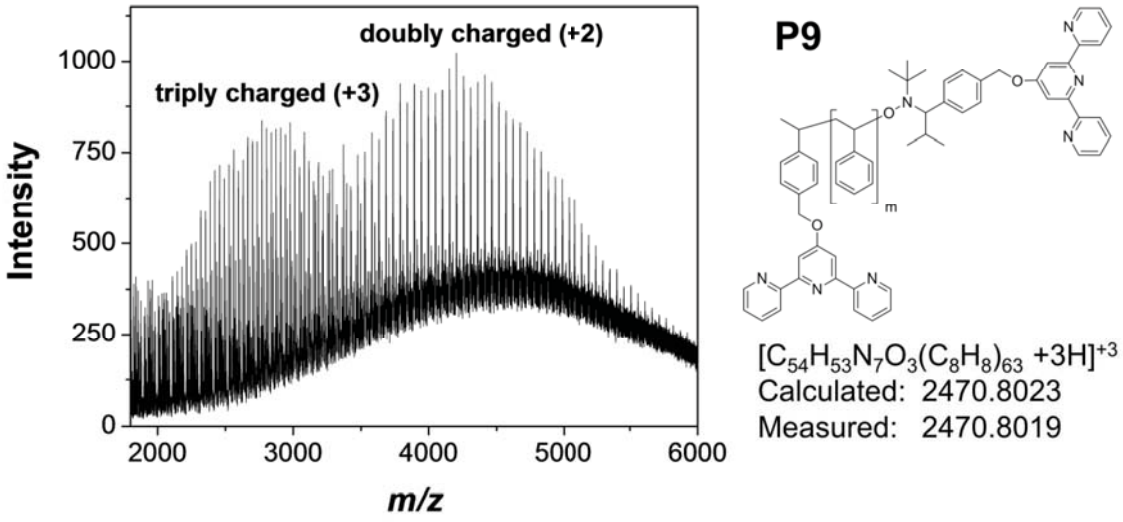


Figure 2.4 ESI-Q-TOF MS spectrum of homotelechelic *bis*-terpyridine containing macroligand (**P9**) (synthesized via NMP of styrene).[39]

In a similar study, Osmium(II) bis-terpyridine complex incorporated copolymer (**P10**) structure was analyzed by ESI-Q-TOF MS in detail (Figure 2.5). The molar mass was determined by ESI-Q-TOF MS and it provided an absolute molar mass of 14,400 g/mol from the deconvoluted spectrum of this polymer (which is in good agreement with the SEC measurements).[40]

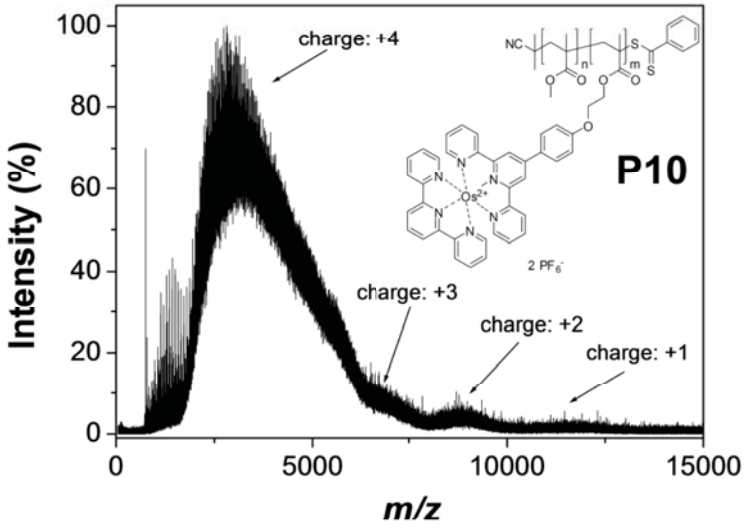


Figure 2.5 ESI-Q-TOF MS spectrum of the Osmium(II) bis-terpyridine complex containing copolymer (**P10**).[40]

In summary, MS is a powerful tool for the structural characterization of synthetic polymers. MS-based techniques are perfectly suitable to determine the absolute molar masses of complex polymeric structures. In addition, MS-based techniques can be also employed to scrutinize monomer units, macromolecular architectures, side chain substituents, end-group functionalities, copolymer compositions and sequences, degradation products, polymerization mechanisms and kinetics as well as degradation products.

3. Comparison of different ionization techniques for polymer characterization

Parts of this chapter have been published: P4) E. Altuntaş, K. Knop, L. Tauhardt, K. Kempe, A. C. Crecelius, M. Jäger, M. D. Hager, U. S. Schubert, *J. Mass Spectrom.* **2012**, *47,* 105-114. P5) E. Altuntaş, A. Baumgaertel, A. Krieg, A. C. Crecelius, U. S. Schubert, *J. Polym. Sci., Part A: Polym. Chem.* **2013**, *51*, 1595-1605. P7) E. Altuntaş, K. Kempe, C. Weber, U. S. Schubert, *Eur. Polym. J.* **2013**, *49*, 2172–2185..

There are numerous ionization techniques which can be used to ionize samples before being introduced into the gas-phase for mass analysis. Some of them can only be used for certain types of analytes. Matrix-assisted laser desorption ionization (MALDI) is the most commonly exploited and simplest MS device to obtain information from synthetic polymers since it generates mostly singly charged species reducing overlaid charge states. MALDI has a great tolerance for contaminations and salts, and allows a MS analysis with high sensitivity and speed. Electrospray ionization (ESI) is a softer analysis technique compared to MALDI and it is more appropriate for labile substances, in particular for polymers with fragile end-groups or supramolecular assemblies held together by means of non-covalent interactions. For ESI-MS analysis, polymer substances have to be completely soluble in polar solvents; in contrast to MALDI analysis, where also partially soluble polymer samples can be analyzed successfully. ESI leads into multiple charging which can cause difficulties in the interpretation of the resulting MS spectra; on the other hand, it also enables the detection of high molar mass substances, which might not be detected in a single charge state due to mass discrimination effects. Furthermore, ESI has a superior ability to comprehensively detect the distinct chemical species present in many polymer samples compared to MALDI.[41] There are plenty of successful examples in literature where both MS techniques assisted polymer scientists to analyze their macromolecular structures.[15, 16, 18, 42-45] However, atmospheric pressure chemical ionization (APCI) is less frequently applied for the analysis of synthetic polymers. In fact, the APCI technique coupled to liquid chromatography (LC) methods open new opportunities for the characterization of polymers. APCI can be applied in particular for low molar mass polymers that are poorly ionizable by the usual methods due to their apolar character.[46] Throughout the presented thesis, these three soft ionization methods (ESI, APCI and MALDI) were exploited to investigate the various polymers with regard to their molecular composition, molar masses (M_n and M_w) and polydispersity index (PDI) values in systematic comparison studies.

The characterization of synthetic polymers is highly dependent on the sample preparation methods and separation technologies prior to MS analysis. Sample preparation methods are incredibly important especially for the characterization of polymer samples via MALDI. Identification of the right choice of solvent, matrix and salt combination is crucial for the successful MALDI analysis of polymers. Unfortunately, the incorrect choice of matrix and salt combination can lead to misleading results particularly for labile/fragile polymeric substances. Solvent and salt used for the MS analysis are similarly important for the characterization of polymers via ESI and APCI techniques. Proposed

ionization processes in ESI, MALDI, and APCI techniques were depicted in Figure 3.1. Some of these processes are still under discussion. Especially in MALDI, the fundamental processes of ion generation and desorption are poorly understood.[47]

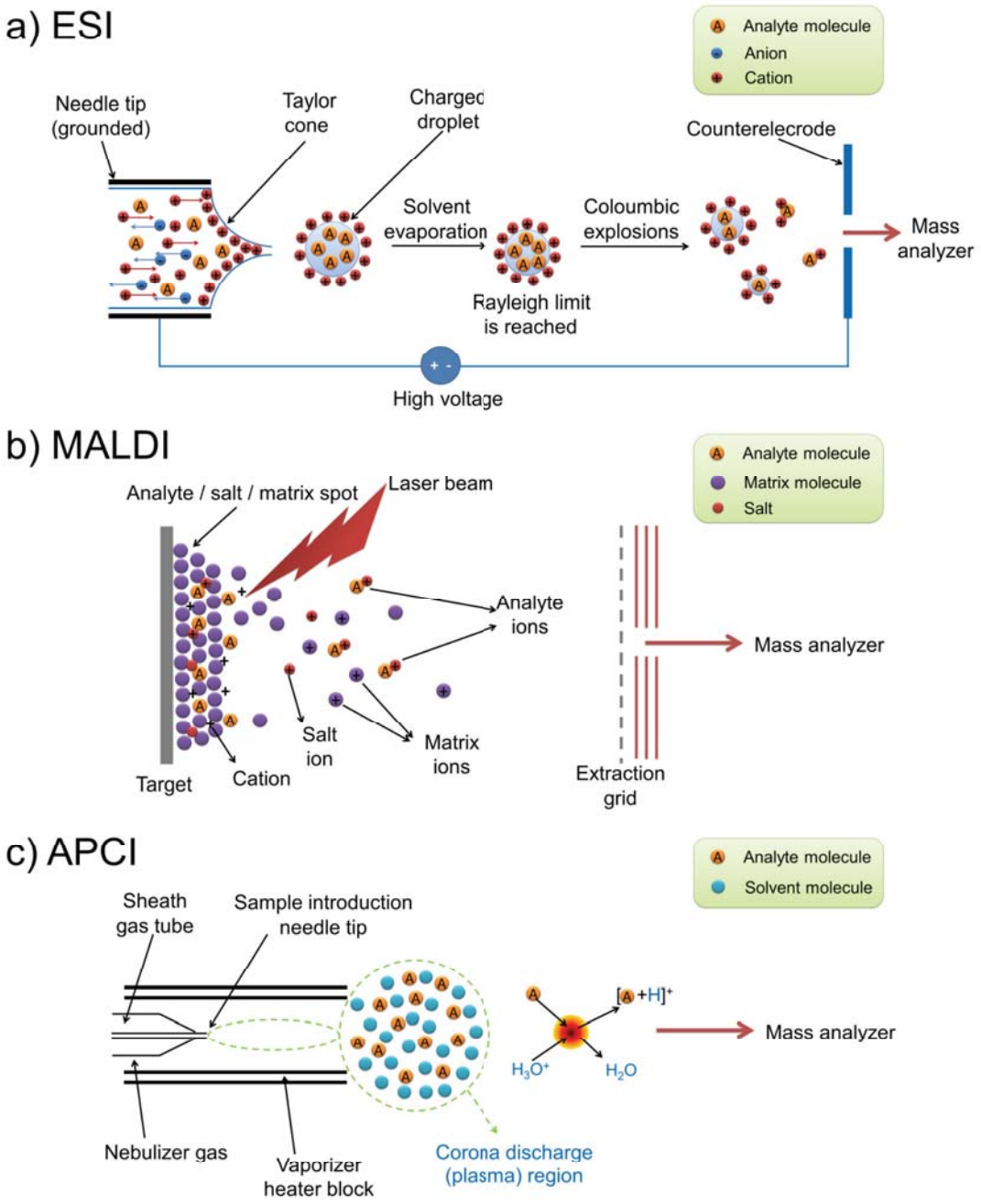


Figure 3.1 Ionization processes in electrospray ionization (ESI) (a), matrix-assisted laser desorption ionization (MALDI) (b), and atmospheric pressure chemical ionization (APCI) (c) [adapted from Ref.[48]].

In the first comparison study, poly(ethylene imine) (PEI) polymers with different initiating and / or terminating end-groups were investigated in order to elucidate the macromolecular structures of these polymers in detail via ESI and MALDI MS. ESI-Q-TOF and MALDI-TOF mass spectra of PEI (**P11**) with hydrogen (H) and hydroxyl (OH) end-groups at the initiating (α) and terminating (ω) chain ends are depicted in Figure 3.2 as a representative example from this study. Both mass spectra reveal the

expected signal spacing correlating to the repeating unit of the investigated PEI (**P11**) (43.04 *Δm/z* for C_2H_5N monomer unit). The expected PEI chains with hydrogen as initiating end-group and hydroxyl as terminating end-group were observed as the main distribution in the ESI-Q-TOF and MALDI-TOF mass spectra (labeled as **A** and **B**). Peak assignments were achieved based on the expected ion *m/z* values and validated by isotopic pattern information. **A** represents the desired protonated distribution $[H(C_2H_5N)_nOH) + H]^+$ and **B** represents the desired sodiated distribution $[H(C_2H_5N)_nOH) + Na]^+$. The minor distribution (**I**) can be explained by inefficient hydrolysis of PEtOx to PEI; because these oligomer series still have one EtOx monomer unit in their backbone $[H(C_5H_9NO)_1(C_2H_5N)_nOH) + H]^+$ or $[H(C_5H_9NO)_1(C_2H_5N)_nOH) + Na]+$ (labeled as **I**), which are present in the both ESI-Q-TOF and MALDI-TOF mass spectra to a very little extent. This observation is supported by ^{1}H NMR spectroscopy revealing a degree of hydrolysis around 99%. The only difference between the ESI-Q-TOF and MALDI-TOF mass spectra arises from some fragments (labeled as **Fr**) which are readily observed in the MALDI-TOF mass spectrum. These fragments were not observed in the ESI-Q-TOF mass spectrum indicating that these fragments were caused by the MALDI-TOF process. In this project, two additional PEI polymers were analyzed in the same way and similar findings were obtained. For the PEI polymer with azide (N_3) terminating end-groups, it was possible to observe the desired PEI polymer structure with the azide moiety as the main distribution in ESI-Q-TOF MS analysis. On the other hand, the main distribution in the MALDI-TOF mass spectrum was the PEI polymer with an amino terminating end-group which was produced by the cleavage of N_2 which occurred during either the MALDI sample preparation or the MALDI process.

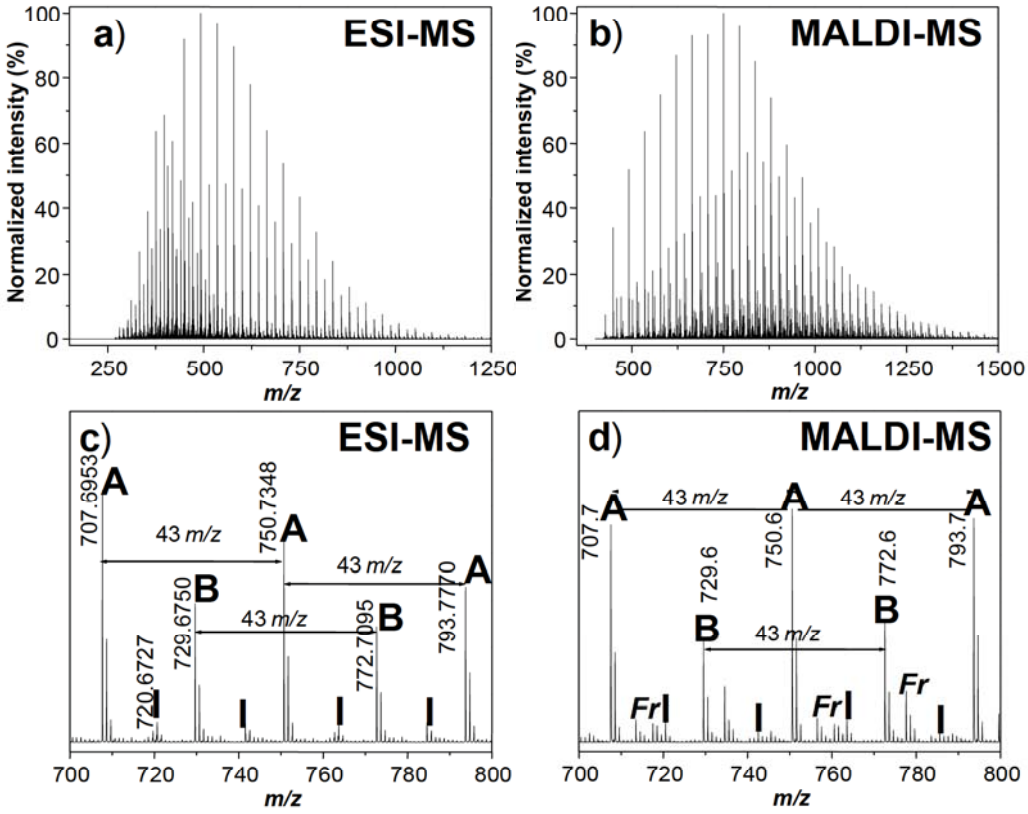
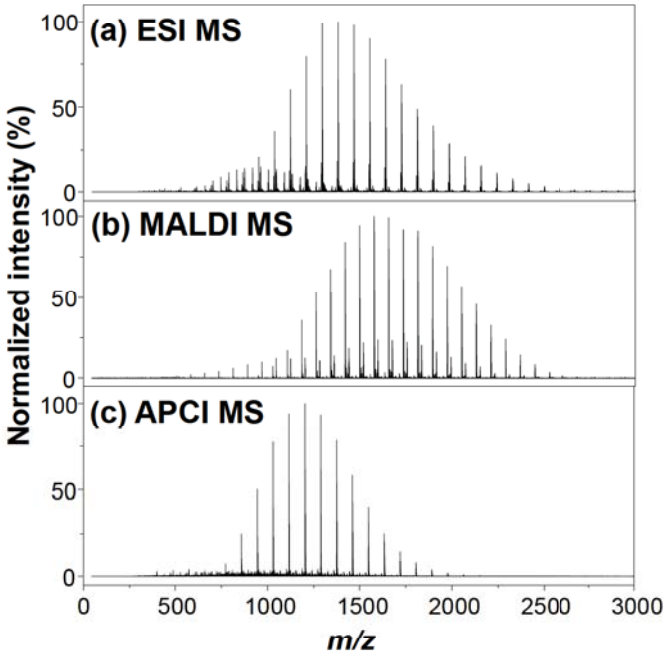
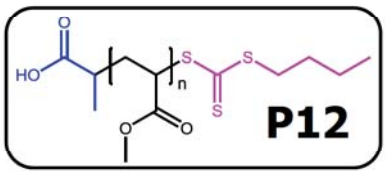


Figure 3.2 (a, b) ESI-Q-TOF and MALDI-TOF mass spectra of a PEI polymer (**P11**) with H and OH end-groups, (c, d) zoom into the ESI-Q-TOF and MALDI-TOF mass spectra of PEI in the range between *m/z* 700 to 800.

In another comparison study, MS analyses of PMA homopolymers synthesized via various CRP techniques were performed for the absolute determination of the molar masses exploiting various ionization techniques (ESI, APCI and MALDI). As it can be easily seen from the obtained results shown in Figure 3.3, the APCI technique does not seem to be a suitable tool for the molar mass determination. The molar mass distributions in MALDI and ESI MS measurements represent the actual molar mass distributions more correctly. Due to the highest tendency of all three techniques to favor the ionization of lower molar mass species, APCI MS should not be considered to provide the true molar mass distributions of the PMA sample (**P12**). On the other hand, APCI allows the application of a wide range of polarities of sample as well as solvent and seems to be complementary to ESI and MALDI. Moreover, the coupling of the APCI instruments to various LC systems is possible (SEC, reversed-phase and normal-phase LC).[46] This combination enables the investigation of synthetic polymers after separation up to certain molar mass values. Therefore, the investigation of synthetic polymers with APCI and complementary tandem mass experiments to elucidate fragmentation processes will provide crucial information in future LC-APCI MS and MS/MS studies.





	Mn (g/mol)	Mw (g/mol)	PDI
SEC	1,850	2,140	1.16
ESI	1,579	1,644	1.04
APCI	1,256	1,316	1.05
MALDI	1,956	2,042	1.04

Mn (^{1}H-NMR)	1,786 g/mol (DP~18)

Figure 3.3 (a) ESI-Q-TOF, (b) MALDI-TOF and (c) APCI-Q-TOF mass spectra of PMA homopolymer (**P12**) synthesized via the RAFT polymerization technique.

All three MS techniques were also exploited to investigate the macromolecular structures of the PMA polymers in the same study. The main distribution belongs in the most cases to the desired polymer structure and a sodium cation, which is most probably leftover from the polymerization. The smaller signal series can be explained by already fragmented polymer chains, which corresponds to closed-shell fragments containing the initiating (α) end-group produced by consecutive dissociation of the primary radical ion series. This series were observed in the MALDI analysis; on the other hand, the fragments were not observed in the ESI and APCI mass spectra. These results suggest that this series were produced by fragmentation of end groups during the MALDI process and are not a side

product from the polymerization reaction. In general, the number of chain end-groups detected is higher by ESI and APCI than by MALDI MS in most of the cases.

In a similar study, eight PEtOx polymers with different end-groups were investigated using the ESI, APCI and MALDI MS techniques to obtain information of the molar masses and molecular compositions. ESI, APCI, and MALDI MS spectra of a representative PEtOx polymer (**P13**) are illustrated together with enlarged regions between *m/z* 1,000 to 1,200 in Figure 3.4. ESI MS provided multiply charged species (+2 and +3) in the lower mass region, whereas, APCI and MALDI MS provided only singly charged species due to their different ionization processes. Similar findings were obtained related to the comparison of these three MS techniques on polymer analysis with respect to the molar mass determination and end-group verification.

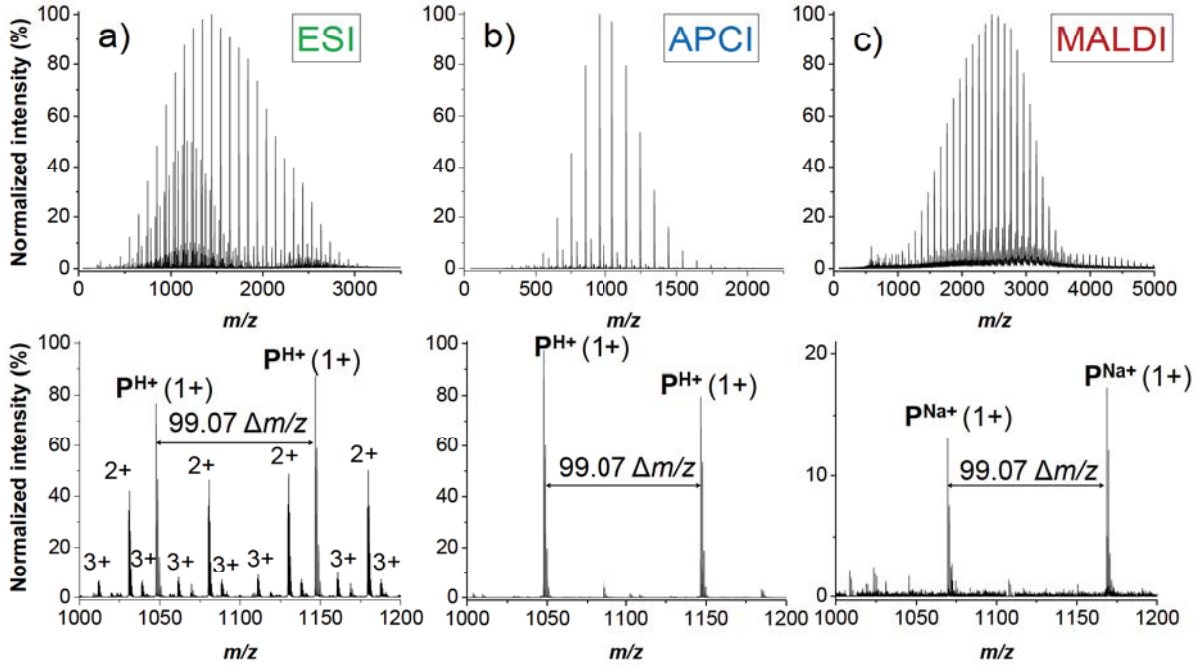


Figure 3.4 ESI MS (a), APCI MS (b) and MALDI MS (c) spectra of a representative poly(2-ethyl-2-oxazoline) (**P13**) (below zoom into the area between 1,000 to 1,200 *m/z* for each spectrum).

In conclusion, the ESI and APCI methods were found to be comparable in respect to the end-group determination and both techniques generally resulted in less in-source fragmentation compared to MALDI technique for the investigated polymer classes (PEI, PMA and PEtOx). Therefore, ESI and APCI should be considered as method of choice in order to identify the maximum number of end-group functionalities within given polymer samples. Furthermore, the ESI and APCI techniques were found to be more efficient in detecting distinct main and side products present in the polymer samples compared to the MALDI technique. However, in order to obtain molar mass information from larger macromolecules and to gain a complete picture of the polymer sample, MALDI should be used as the preferred technique.

4. Tandem mass spectrometry of polymers

Parts of this chapter have been published: P2) E. Altuntaş, K. Kempe, A. Crecelius, R. Hoogenboom, U. S. Schubert, *Macromol. Chem. Phys.* **2010**, *211*, 2312-2322. P4) E. Altuntaş, K. Knop, L. Tauhardt, K. Kempe, A. C. Crecelius, M. Jäger, M. D. Hager, U. S. Schubert, *J. Mass Spectrom.* **2012**, *47,* 105-114. P5) E. Altuntaş, A. Baumgaertel, A. Krieg, A. C. Crecelius, U. S. Schubert, *J. Polym. Sci., Part A: Polym. Chem.* **2013**, *51*, 1595-1605. P8) A. Baumgaertel, E. Altuntaş, K. Kempe, A. C. Crecelius, U. S. Schubert, *J. Polym. Sci., Part A: Polym. Chem.* **2010**, *48*, 5533-5540.

Tandem mass spectrometry (MS/MS, MS^2 or MS^n) includes multiple stages of mass spectrometry experiments in order to form fragments from the studied substances and to analyse the obtained fragment ions associated with particular structures. Many biological samples such as proteins, metabolites, lipids and genes can be analysed by MS^n in order to gain sequence information. MS^n techniques can also be utilized for the characterization of polymers to elucidate further information about the macromolecular structures and to differentiate isobaric and isomeric species. A simple workflow for the characterization of synthetic polymers via tandem MS analysis is depicted in Figure 4.1.

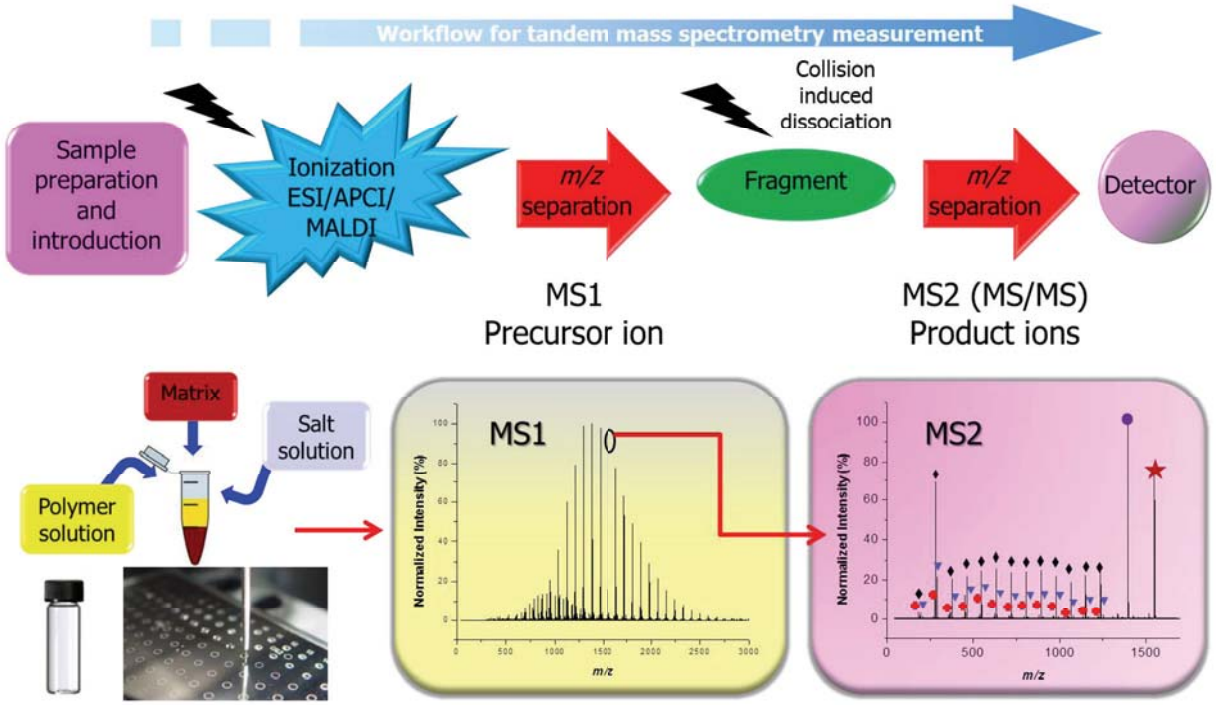


Figure 4.1 Schematic representation of a workflow for the tandem MS analysis of synthetic polymers.

Subsequent to exploring the potential of the developed single dimensional MS analysis methods, several important polymer classes such as poly(2-oxazoline)s (POx), poly(methylacrylate)s (PMA), and poly(ethylene imine)s (PEI) have been scrutinized in this thesis in order to investigate the effect of different end-groups on the polymer fragmentation mechanisms via various MS/MS techniques utilizing electrospray ionization (ESI), matrix-assisted laser desorption ionization (MALDI) and

atmospheric pressure chemical ionization (APCI). As a result, a significant influence of the end-groups on the fragmentation mechanisms of all the studied polymer classes and gathered significant structural information about these polymers from MS/MS investigations could be observed. In general, our scientific objective was to build tandem MS libraries to provide basic knowledge on the fragmentation mechanisms of synthetic polymers to the polymer science community and to use this knowledge for certain applications of these polymers.

In the first detailed tandem MS study, various POx polymers with different side groups (*i.e.* methyl, ethyl, 1-ethylpentyl, phenyl, *o*-difluorophenyl and *p-tert*-butylphenyl) were investigated via ESI-Q-TOF MS/MS to attain knowledge about their fragmentation mechanisms. POx with the same initiating and terminating end-groups were selected in order to examine the effect of the side chains on the fragmentation pathways. As a result, it was found that the nature of the side-chain substituents influences the fragmentation mechanism undergone during the MS/MS process. ESI-Q-TOF MS/MS of the POxs revealed that the main fragmentation mechanism was the depolymerization mechanism (which can be called an unzipping mechanism or monomer evaporation). The elimination of small molecules such as ethene and hydrogen in their fragmentation patterns were also observed (1,4-hydrogen and ethene eliminations), which are partially dependent on the side groups. Also, a McLafferty rearrangement can be a possible fragmentation route for these polymers. Moreover, the side group elimination was only observed for poly(2-alkyl-2-oxazoline)s, but not for poly(2-aryl-2-oxazoline)s.[37, 49]

In a subsequent study, PEtOx polymers with different initiating and terminating end-groups were also investigated to explore the effect of the end-groups on the fragmentation pathways. ESI, APCI and MALDI tandem MS techniques were utilized in order to enable a detailed comparison and to gain the necessary knowledge about the fragmentation pathways. As depicted for a representative PEtOx polymer (**P14**) in Figure 4.2, ESI and APCI MS/MS provided the same fragmentation products, whereas MALDI MS/MS delivered a large number of additional fragmentation products. Tandem MS analysis of the PEtOxs via MALDI MS/MS revealed the elimination of small molecules and McLafferty rearrangement was also observed as already found in earlier studies. On the other hand, the main fragmentation mechanism observed from the ESI and APCI MS/MS studies was the depolymerization mechanism. One reason for this observation is most likely the different cationization agent utilized. Another reason for the differences between the tandem MS techniques is probably the selective ionization in the employed ESI and APCI processes. This situation can mainly be attributed to a favorable ionization of lower abundance species (isomeric side product species) when using ESI and APCI MS. The fragmentation of the side product (with an amino / ester end-group $[C_3H_3(C_5H_9NO)_{n-1}O_2C_5H_{10}N]$) is significantly easier compared to the fragmentation of the main product (with a hydroxyl end-group $[C_3H_3(C_5H_9NO)_nOH]$), which requires higher energies to cleave the bond between the polymer backbone and the end-group functionality. This observation was valid for all PEtOx polymers with a hydroxyl end-group functionality.

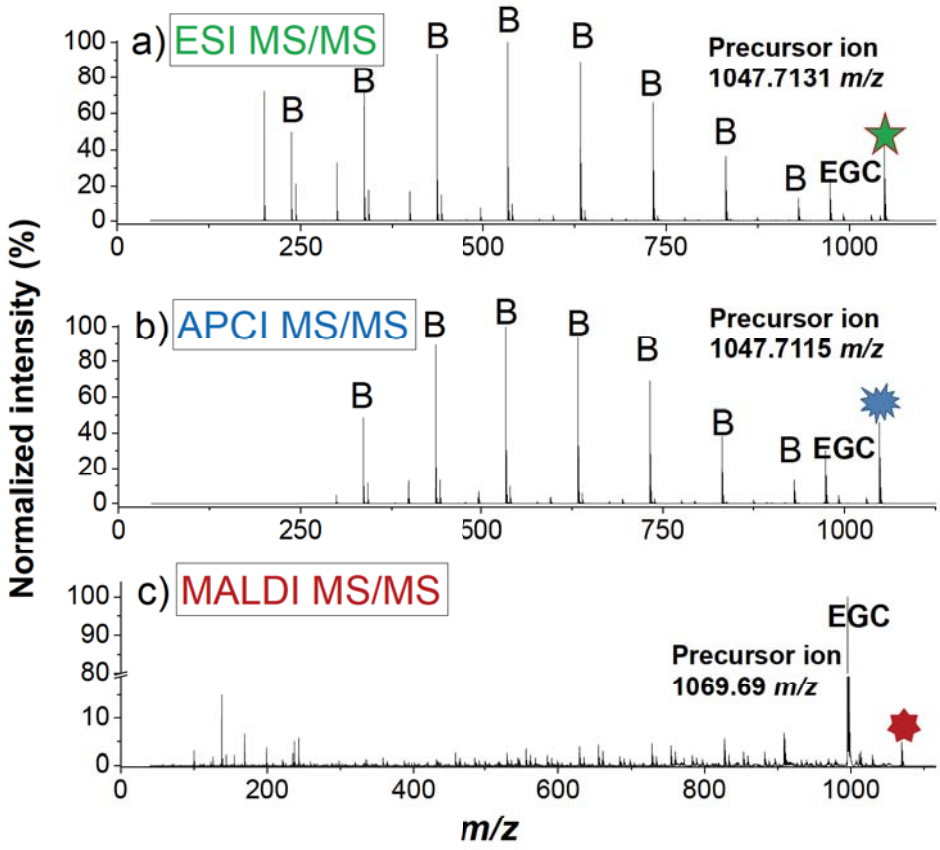


Figure 4.2 ESI (a), APCI (b) and MALDI (c) tandem mass spectra of a representative poly(2-ethyl-2-oxazoline) sample (**P14**) (EGC: end-group cleavage, B: fragmentation products from depolymerization process).[50]

In a succeeding study, PEI samples were also analyzed by ESI-Q-TOF and MALDI-TOF MS/MS to gain information about their fragmentation pathways. ESI-Q-TOF and MALDI-TOF tandem mass spectra of a representative PEI sample (**P11**) were shown in Figure 4.3. The tandem mass spectra contain four major distributions of fragment ion series ($\mathbf{A}^{X}_{n}$, $\mathbf{A}^{Y}_{n}$, $\mathbf{B}^{X}_{n}$, and $\mathbf{B}^{Y}_{n}$) and some minor distributions of fragment ion series ($\mathbf{J}^{VV}_{n}$, $\mathbf{J}^{VA}_{n}$, and $\mathbf{J}^{AA}_{n}$). As possible fragmentation mechanisms, a 1,2-hydride shift with a charge-remote rearrangement via a four-membered cyclic transition state as well as charge-induced fragmentation reactions were proposed according to the obtained fragmentation products from the protonated parent peaks. In addition, heterolytic and homolytic cleavages were proposed as alternative fragmentation pathways. The possible fragmentation mechanism resulted in the formation of two different functionalities at the terminal chain ends. Superscripts X and Y are used to represent ions containing initiating (α) and terminating (ω) end groups, respectively ($\mathbf{A}^{X}_{n}$, $\mathbf{A}^{Y}_{n}$, $\mathbf{B}^{X}_{n}$, and $\mathbf{B}^{Y}_{n}$). In this proposed mechanism, CID spectra of protonated PEIs occur through a charge-remote process in which the ionizing proton does not play an active role in the cleavage. The main fragment series ($\mathbf{A}^{X}_{n}$, $\mathbf{A}^{Y}_{n}$, $\mathbf{B}^{X}_{n}$, and $\mathbf{B}^{Y}_{n}$) contain one of the original end groups and new end groups (-NH_2 or -$CH{=}CH_2$), which are formed by the proposed charge-remote fragmentation mechanism, and the *m/z* difference between two sequential fragmentation ions within one series corresponds to 43.04 *m/z* units representing the mass of one repeating unit of PEI. The most abundant fragment ions series ($\mathbf{A}^{X}_{n}$, $\mathbf{A}^{Y}_{n}$, $\mathbf{B}^{X}_{n}$, and $\mathbf{B}^{Y}_{n}$) apparently result from this proposed mechanism, whereas the less abundant fragment ion series correspond to internal fragments ($\mathbf{J}^{VV}_{n}$, $\mathbf{J}^{VA}_{n}$, and $\mathbf{J}^{AA}_{n}$) produced by a more

complex fragmentation process, where this charge-remote fragmentation occurs in both sides of the oligomer chains. Internal fragments with a variety of end groups were named as J_n species with different superscripts which indicate their end groups such as vinyl/amine ($J^{VA}{}_n$), vinyl/vinyl ($J^{VV}{}_n$), and amine/amine ($J^{AA}{}_n$). It must be noted that the ions of the main fragment series can result from both charge-remote and charge-induced dissociation mechanisms, but it is difficult to evaluate the extent of fragment ions from each pathway. Dissociation via competing charge-induced cleavage leads to similar fragments. Charge-induced cleavage of C-N bond followed by an energetically favorable 1,2-hydride shift could be an alternative to the charge-remote fragmentation.[28]

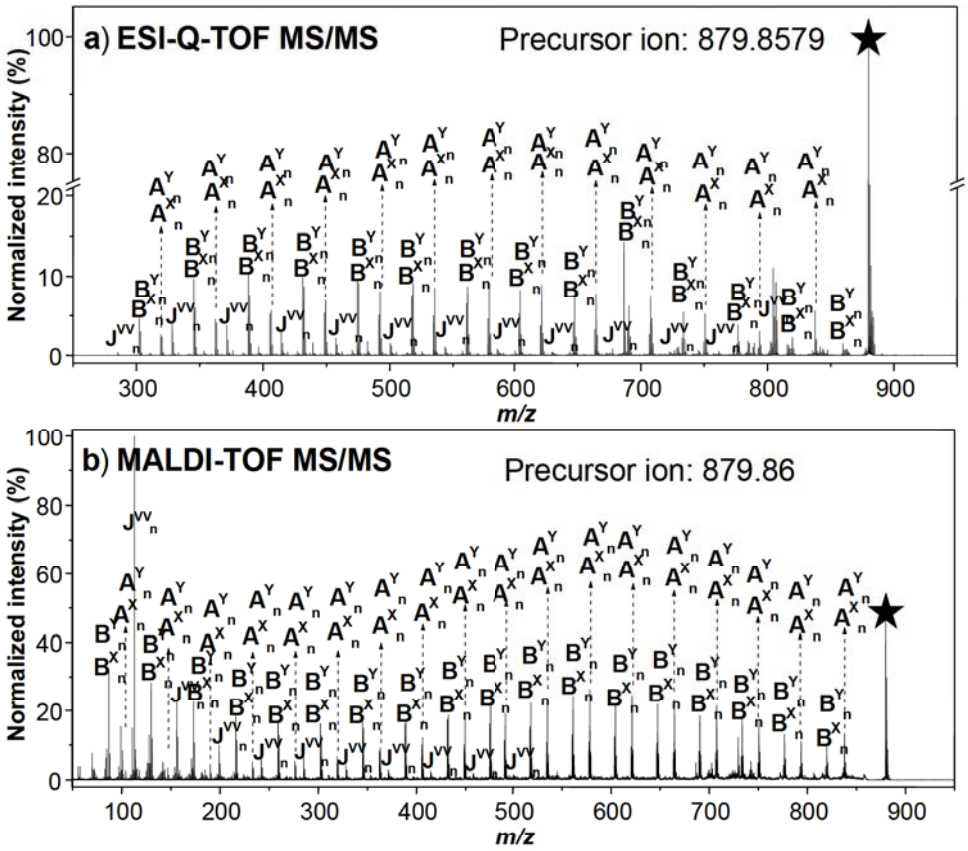


Figure 4.3 (a, b) ESI-Q-TOF and MALDI-TOF/TOF tandem mass spectra of the protonated PEI (**P11**) parent peak with 20 repeating units marked with a star $[H(C_2H_5N)_{20}CH + H]^+$ (*m/z* 879.8579).

The tandem mass spectra of the protonated and the sodiated PEI precursor ions revealed some differences. As depicted in Figure 4.4, a comparison of the ESI-Q-TOF tandem mass spectra of the protonated PEI precursor ion $[M+H]^+$ (*m/z* 879.8579) and the sodiated PEI precursor ion $[M+Na]^+$ (*m/z* 901.8476) shows that the sodiated precursor ions are more stable than the protonated precursor ions. The protonated species could easily be fragmented at low collision energies (20 to 60 eV), while the sodiated species could only be fragmented to a small extent even at higher collision energies (60 to 200 eV). This indicates that sodiated ions are more stable towards collisional dissociation; therefore, there is a need to use higher collision energies for obtaining fragments from sodiated species.

The fragmentation mechanisms of collisional dissociation for sodiated and protonated species are also different. For the sodiated parent peaks, possible fragmentation pathways like the 1,4-hydrogen elimination were proposed and the formed structures (B^X_n, B^Y_n, C^X_n, C^Y_n, D^X_n, and D^Y_n) were correlated to the obtained masses of the fragmentation products (Figure 4.4).

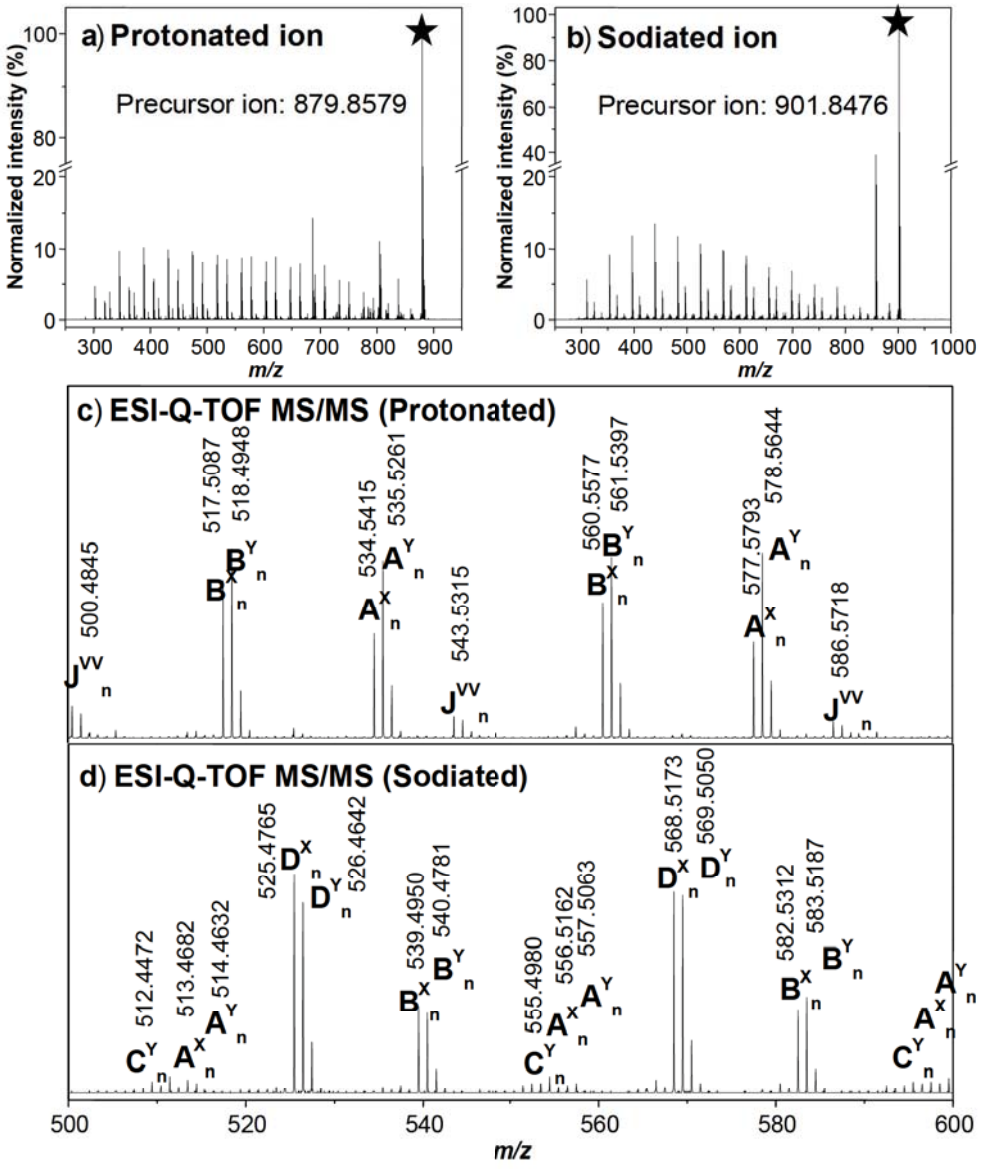


Figure 4.4 (a, b) ESI-Q-TOF tandem mass spectra of the protonated $[H(C_2H_5N)_{20}OH + H]^+$ (*m/z* 879.8579) and sodiated $[H(C_2H_5N)_{20}OH + Na]^+$ (*m/z* 901.8476) PEI (**P11**) parent peak with 20 repeating units and (c, d) zoom into the ESI-Q-TOF tandem mass spectra of the protonated and sodiated PEI in the range between *m/z* 500 to 600.

In another study, a detailed characterization of PMA polymers using different MS/MS techniques (ESI, APCI, and MALDI MS/MS) was performed to provide insights into the macromolecular structure (Figure 4.5). The labile end-groups were determined based on the different fragmentation behavior in CID process. The fragment ions in the upper mass region, which are closer to the precursor ion, provided the best information about the end-group cleavages. Five main fragment series could be observed in the low molar mass region of the MS/MS spectra independent of the polymerization

technique. The letter **a** or **b** indicates these fragments retain the initiating chain-end and the subscripted **n** value provides the number of repeating units contained in the fragment. Internal fragmentation product ions (both original end-groups are missing) arising from further dissociations were named with the middle letter from the alphabet such as **K** and **J**. Beside the series $\mathbf{a_n}$ and $\mathbf{b_n}$ (from depolymerization), fragment series $\mathbf{a_{nb}}$, $\mathbf{b_{nb}}$, $\mathbf{K_n}$ and $\mathbf{J_n}$ could be identified. A possibility for the formation of the fragment series $\mathbf{b_{nb}}$ is starting from the secondary radical ions $\mathbf{b_n}\bullet$, followed by a β C-C bond scission to release the polymer side group and a hydrogen movement to the polymer chain-end to form the closed-shell fragment. This mechanism is similar to the already explained fragmentation pathway for the series $\mathbf{a_{nb}}$. However, the dissociation of the side groups requires significantly more energy than dissociation via monomer evaporation. Therefore, $\mathbf{a_{nb}}$/ $\mathbf{b_{nb}}$ series have very low relative abundances due to the energetically more favorable depolymerization process. Fragment series with an intact ω end-group could not be observed in the tandem MS spectra because of the fragile end-groups.

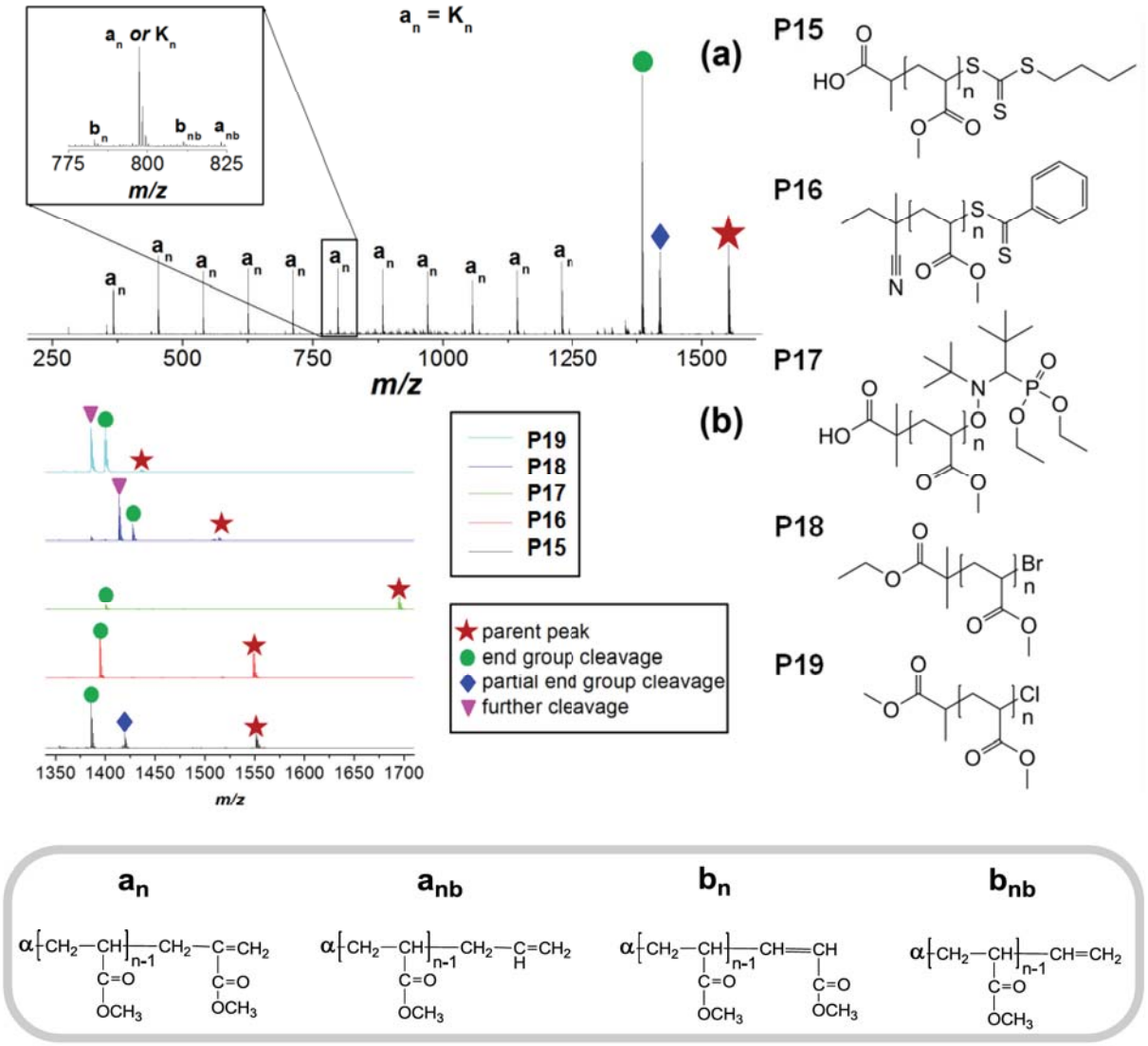


Figure 4.5 (a) ESI-Q-TOF tandem mass spectra of **P15** (*m/z* 200 to 1700) and (b) the comparison of the fragmentation behavior due to the different starting and end-groups of all investigated homopolymers **P15-P19** (upper mass region).

In addition, several POx block copolymers were analyzed in detail by MALDI-TOF MS/MS and ESI-Q-TOF MS/MS. This study confirmed the block copolymer structure and elucidated successfully the fragmentation behavior within the polymer chain. In particular, the ESI-Q-TOF MS/MS data have provided important structural information on copolymer sequence due to the depolymerization mechanism of the ESI-generated copolymer ions. The identification of copolymer sequences was easily achieved by monitoring the depolymerization mechanism of the POx block copolymers (Figure 4.6).

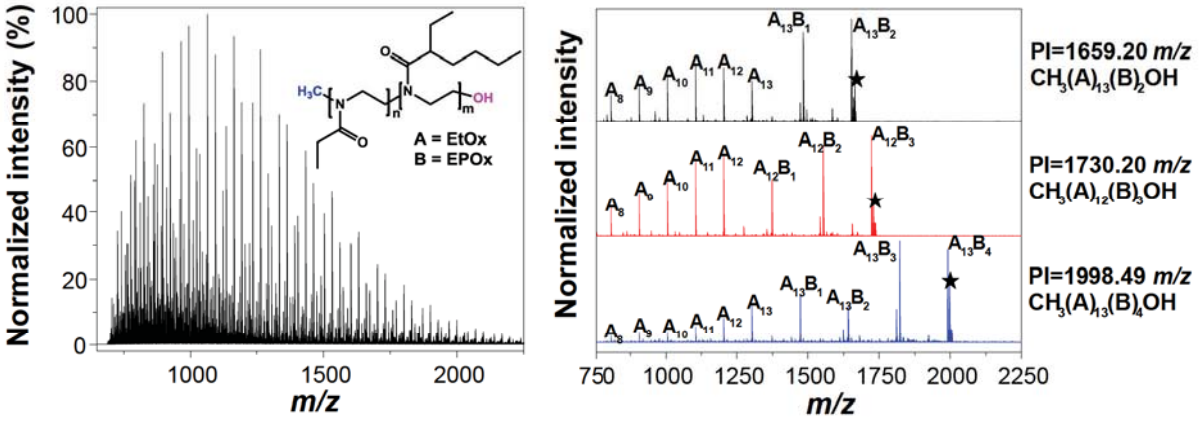


Figure 4.6 ESI-Q-TOF MS and MS/MS spectra of p(EtOx-*b*-EPOx) copolymer (**P8**).

Overall, throughout the presented thesis, tandem MS technique was successfully utilized for the detailed characterization of various synthetic polymers (various homopolymers, copolymers and other polymeric architectures). Valuable structural information was obtained to elucidate the fragmentation behavior of these polymer classes and this knowledge can be used by polymer community in the future to analyze similar polymer classes. Sequential information about POx copolymers were achieved by the evaluation of the fragmentation products obtained via MS/MS analysis. In addition, the usefulness of MS/MS technique for application in polymer science could be shown.

5. Energy-dependent collision-induced dissociation for analysis of polymers and supramolecular substances

Parts of this chapter have been published: P3) E. Altuntaş, A. Winter, A. Baumgaertel, R. M. Paulus, C. Ulbricht, A. C. Crecelius, N. Risch, U. S. Schubert, *J. Mass Spectrom.* **2012**, *47*, 37-43. P6) E. Altuntaş, C. Weber, U. S. Schubert, *Rapid Commun. Mass Spectrom.* **2013**, *27*, 1095–1100.

MS^n has been proven to be a powerful tool for the structural characterization of synthetic polymers and supramolecular compounds. It represents an important technique to complement the existing analytical methods that face the complexity of these substances. As explained in Chapter 4, MS^n experiments can be performed upon precursor ions of interest in order to gain further structural information from the resulting fragmentation patterns. However, there is still a need for methodologies to further elucidate the structural features. One approach is to develop standardized MS^n methods that allow the derivation of structural information of macromolecules from the fragmentation patterns. In order to accomplish this task a good understanding of the fragmentation behavior is required in different excitation conditions and as a function of their composition. This requires the analysis of these macromolecules via energy-dependent CID experiments to investigate the dependency of the fragmentation patterns as a function of collision energy.

The ESI-MS technique can be utilized for this kind of studies, since it is a very sensitive, accurate, and soft ionization technique, and suitable for the study of various macromolecules with minimal sample consumption. Additionally, this technique generally allows the ionization of different macromolecules with little or no fragmentation. Therefore, ESI-MS^n can be employed for the investigation of the amount of energy necessary to drive the dissociation reaction fast enough to observe fragments, and to calculate characteristic collision energy (CE_{50} or CCE)[25] and threshold collision energy[51] values for these substances. The threshold collision energy can be considered as the collision energy value sufficient to produce fragments in the collision cell and the relative binding strengths of supramolecular complexes can be investigated by utilizing these special energy values. The CE_{50} is the collision energy required to obtain 50% fragmentation from the analyzed substances. CE_{50} was already used as a tool to distinguish different classes of polymers in the literature such as polyethers, polymethacrylates, polyesters, and polysaccharides.[27, 52-57] Obtaining survival yield (SY) curves from the energy-dependent ESI-MS^n data offers a useful way of presenting all the information of the CID processes undergone by a specific type of polymer ion. CE_{50} values can be easily determined from the SY curves and these values could discriminate between different compounds. The SY method reveals insights of the energy requirements of the fragmentation pathways of polymers. Therefore, it is a promising way for identification and discrimination of polymers with different functionalities or backbones.

In this part of the thesis, the energy-dependent CID experiments performed to analyze sodiated PEtOxs with different functional end-groups (Figure 5.1) by ESI-Q-TOF MS/MS will be discussed. The fragmentation mechanisms of electrospray-generated POx ions have already been examined in Chapter 4. Here, the main aim was to study the detailed fragmentation patterns of various PEtOx with different end-groups using ESI-Q-TOF MS/MS by varying applied collision energy values (0 to 200 eV) and to create SY curves from the data obtained via these energy-dependent CID experiments.

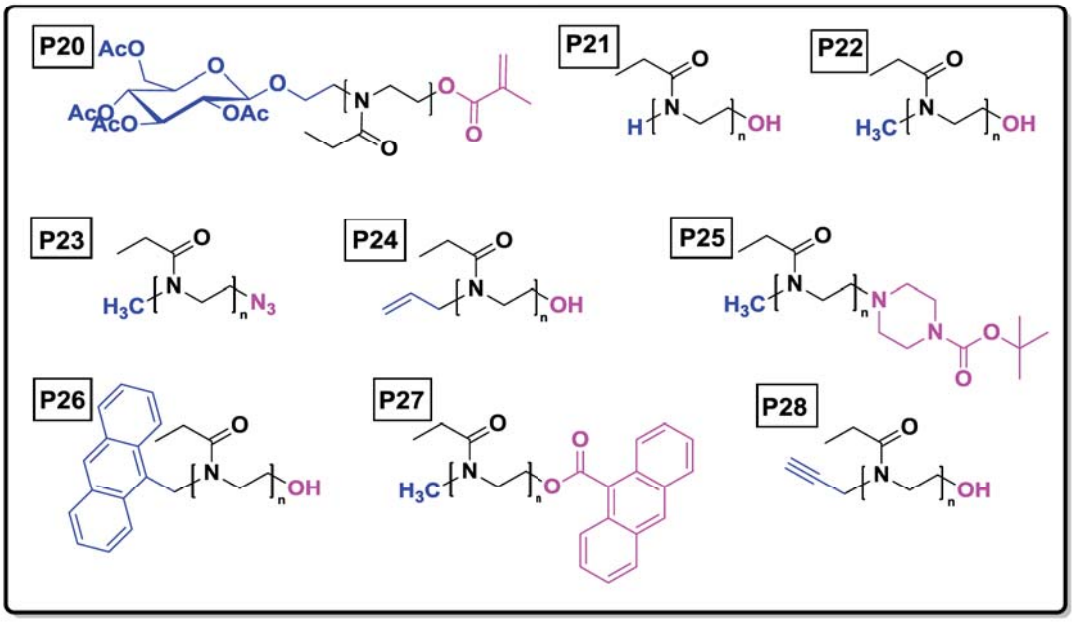


Figure 5.1 Schematic representation of the structures of the studied poly(2-ethyl-2-oxazoline)s with different end-groups (**P20**-**P28**).

Figure 5.2 demonstrates the fragmentation behavior of the sugar decorated PEtOx polymer **P20** under different collision energy values (10 to 100 eV). In general, PEtOxs with a fragile end-group such as esters can be easily fragmented by the depolymerization process (*i.e.* representative example **P20**). In the beginning of the fragmentation process, the loss of the labile end-group was observed with consecutive monomer evaporation (unzipping or depolymerization mechanism). Upon the increase of the applied collision energies, this depolymerization mechanism can be easily followed down to one monomer unit with the initiating end-group. On the other hand, for PEtOxs with bad leaving end-groups such as hydroxyl end-groups, a large number of further fragment ion series can be observed due to other type of backbone cleavages (*e.g.* 1,4 hydrogen or ethylene elimination out of the backbone, McLafferty rearrangement and side group eliminations). For the latter PEtOxs, the depolymerization mechanism was not the main fragmentation pathway. These findings reveal that the fragmentation mechanism is mainly influenced by the quality of ω end-groups as the leaving group. On the other hand, the influence of the α end-groups on the fragmentation mechanism was less pronounced, which supports the hypothesis that indeed a depolymerization is observed for good leaving groups which easily can form a stable oxazolinium end-group.[58]

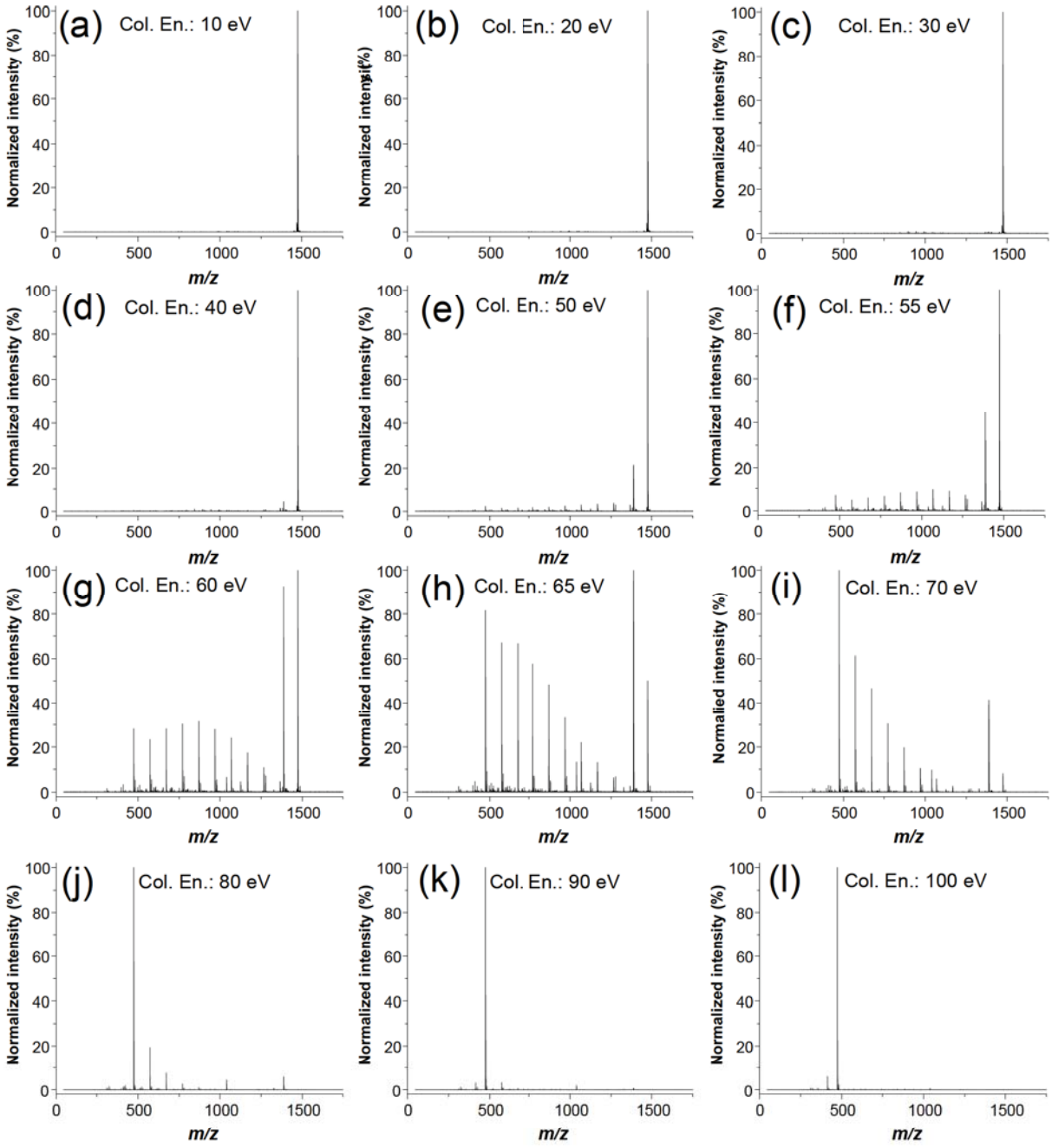


Figure 5.2 ESI MS/MS spectra of PEtOx (**P20**) at *m/z* 1473.82 recorded at different collision energies (10 to 100 eV).

The survival yield (SY) curves of various sizes of the studied PEtOxs were drawn by plotting the SY of the precursor ion against the applied collision energy, and the collision energy values related with the characteristic point of 50% precursor ion were determined. Figure 5.3 represents the explanation of this process for the glucose-functionalized PEtOx (**P20**) with 10 monomer units. The experimental data points are shown together with the corresponding sigmoidal fits. The abscissa of the characteristic point at 50% SY of the sigmoidal fit represents the CE_{50} value for the investigated PEtOx (**P20**).[58]

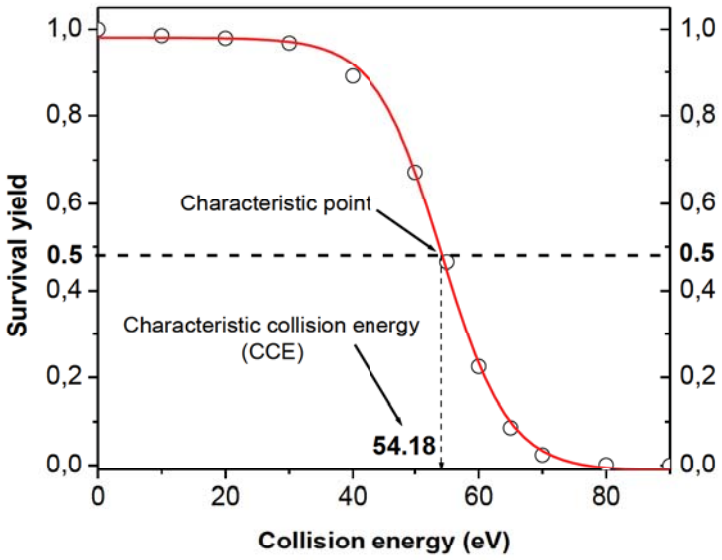


Figure 5.3 Survival yield (SY) curve for glucose-functionalized PEtOx with 10 monomer unit (**P20**).

The CE_{50} values of PEtOxs as a function of the precursor ion mass was calculated and a relationship between the CE_{50} values and the molecular size for all PEtOx polymers was investigated. The findings showed that the collision energy required to achieve 50% fragmentation was linearly dependent on the precursor ion mass (Figure 5.4a). Moreover, the collision energy necessary to promote fragmentation was investigated for PEtOx with different end-groups (**P20-P28**). Upon comparison of the obtained SY curves, one group of polymers can be identified that apparently requires more energy to undergo fragmentation (CE_{50} > 66 eV): **P21**, **P22**, **P24**, **P26** and **P28** (Figure 5.4b). All of these PEtOxs contain a hydroxyl ω end-group and fragment through backbone cleavages instead of the depolymerization mechanism. Among the four other polymers **P20** and **P27** have the lowest CCE values. They have ester ω end-groups, which promote fragmentation via the depolymerization mechanism. Thus, the varying ω end-groups can be categorized according to their CCE values and bond energies: ester < azide < amine << hydroxyl. In turn, the α end-group of the studied PEtOx polymers has no significant influence on the CCE value since all polymers with hydroxyl end-groups have similar CCE values, despite the structural diversity of their α end-groups. However, the influence of the end-group on the CCE is still less significant than the influence of molar mass of precursor ion.[50]

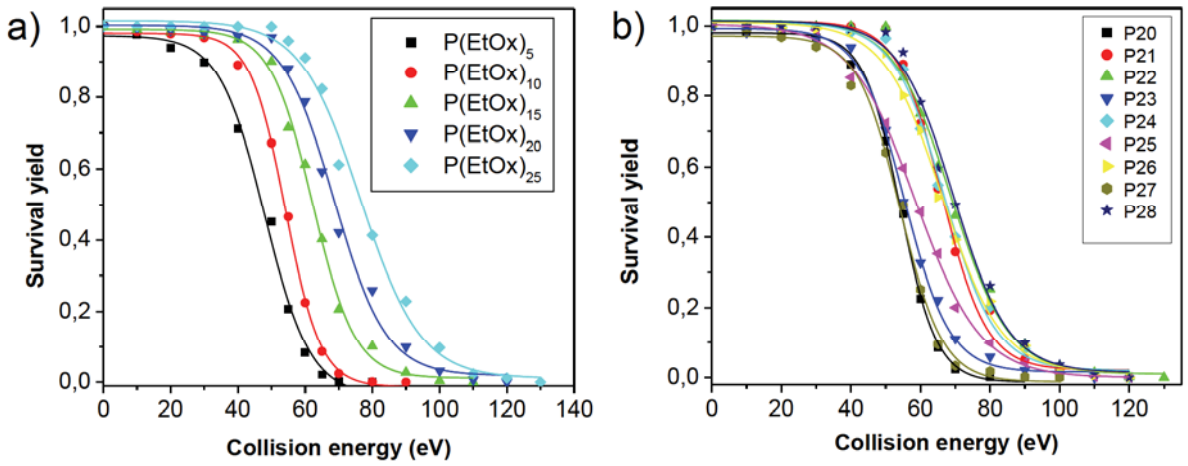
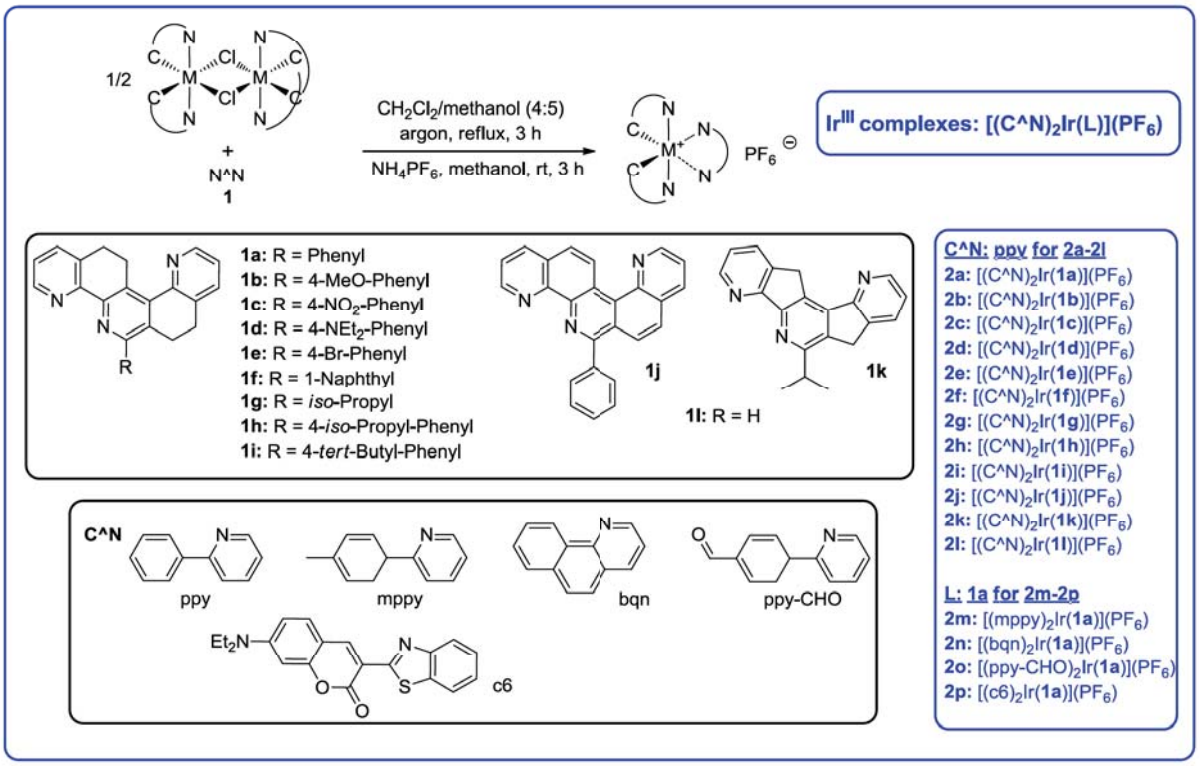


Figure 5.4 (a) SY curves for different molar masses of sodiated **P20** (PEtOx polymer with DP = 5, 10, 15, 20 and 25, respectively), and (b) SY curves of various PEtOx with different end-groups (**P20** to **P28**).

In a different study, the characterization of a library of cationic bis-cyclometalated Ir^{III} complexes were performed using ESI-Q-TOF MS/MS in order to elaborate the relative binding strength of these complexes in the gas phase utilizing variable collision energies. Scheme 5.1 shows the synthesis of the investigated heteroleptic Ir^{III} complexes (**2**). It was possible to estimate the complex stabilities in the gas phase in dependence of the coordinated ligand(s) simply by defining the threshold collision energies and the point of complete complex dissociation.[51]



Scheme 5.1 Schematic representation of the studied heteroleptic iridium(III) (**2**) complexes.

The threshold collision energy can be considered as the collision energy value sufficient to produce fragments from these complexes (**2**) in the collision cell and the complete complex dissociation can be defined as the [Fragment/Complex] ratio ≥ 10. The relative gas phase stabilities of **2** can be determined by comparing the applied collision energies. The ratio of signal intensities of [Fragment/Complex] increased continuously with increasing collision energy utilized for the MS^n analysis, indicative for the fragmentation/destruction of the complex.

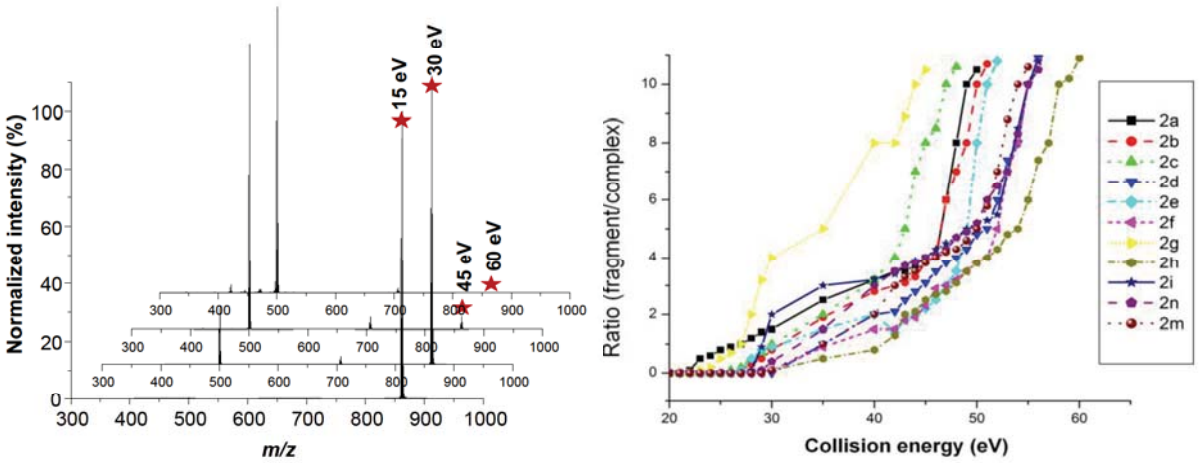


Figure 5.5 ESI-Q-TOF MS/MS spectra of complex $[(ppy)_2Ir(1a)]^+$ (**2a**) at different collision energies and energy-variable CID curves for the investigated Ir^{III} complexes **2**.

Figure 5.5 depicts the fragmentation profile of a representative complex sample **2a** in the tandem MS process as a function of the applied collision energy values and the corresponding degradation curves reflecting this characteristic trend for all complexes. The Ir^{III} complexes (**2**) exhibited a different degradation behavior depending on the nature of the coordination sphere (*i.e.* C^N and S-tpy ligand).

All in all, it was possible to gain additional information about the fragmentation behavior and the stabilities of various macromolecules from energy dependent CID experiments. SY curves were shown as a useful tool to correlate the macromolecular structure of PEtOxs to their fragmentation behavior. In addition, energy dependent CID experiments were also performed successfully to analyze the gas-phase stabilities of various metal complexes.

6. Hyphenated techniques for polymer characterization

Parts of this chapter have been / will be published: P9) A. Baumgaertel, C. Weber, N. Fritz, G. Festag, E. Altuntaş, K. Kempe, R. Hoogenboom, U. S. Schubert, *J. Chromatogr. A* **2011**, *1218*, 8370-8378. P10) A. Baumgaertel, E.Altuntaş, U. S. Schubert, *J. Chromatogr. A* **2012**, *1240*, 1-20.

As already emphasized in Chapter 1, synthetic polymers have complex structures. As a consequence, advanced sample preparation and separation techniques have to be utilized prior to MS analysis in order to achieve an effective analysis of these complex polymeric systems. The structural complexity of synthetic polymers can occur from the variations in chain length, functionality, composition and architecture. This complexity can be illustrated in the different aspects such as the molar mass distribution (MMD), the chemical composition distribution (CCD), the functionality type distribution (FTD) and the molecular architecture distribution (MAD).[59] There are several chromatography techniques which are sensitive towards a specific type of heterogeneity. In order to elucidate the actual molecular structure of synthetic polymers, it is necessary to combine these separation techniques receptive with respect to different heterogeneities (so-called two-dimensional (2D) chromatography). A number of chromatography techniques such as size-exclusion chromatography (SEC), liquid adsorption chromatography (LAC), liquid chromatography at critical conditions (LCCC), gradient polymer elution chromatography (GPEC), and temperature gradient interaction chromatography (TPIC) can be coupled in order to gain information about different properties, like molar mass, chemical composition or functionality in one experiment.[60] One common approach for the detailed polymer characterization via 2D chromatography is the coupling of LCCC and SEC techniques (Figure 6.1). LCCC is a technique which separates synthetic polymers according to their chemical composition irrespective of their molar masses; on the other hand, SEC separates synthetic polymers according to their molar masses (hydrodynamic volume). The LCCC-SEC coupling opens up new possibilities to characterize different homopolymers, polymer blends, block and random copolymers, as well as star-shaped polymers.[61-67] However, above mentioned chromatography methods have certain limitations and drawbacks, even 2D analysis techniques are sometimes not sufficient to solve the problems in the characterization of complex polymer systems completely. For that reason, there is a need for the hyphenation of chromatography techniques to MS-based techniques. By this way, one can identify every fraction from the 2D analysis using MS and MS/MS techniques by acquiring additional structural information. For this purpose, MALDI MS can be coupled offline by means of various spraying or spotting techniques.[68, 69] On the other hand, the 2D-LC setup can be easily coupled online to an ESI MS system.[70-73] Ultimately, MS and MS/MS analysis of polymers can generally provide useful information about their monomer units, end- and side-group functionalities, block lengths and composition, architectures as well as copolymer sequences. In particular, MS/MS can be employed to verify the peak assignments, to identify individual end-groups, to differentiate isobaric and isomeric species and to analyze the macromolecular architectures of polymers in detail.

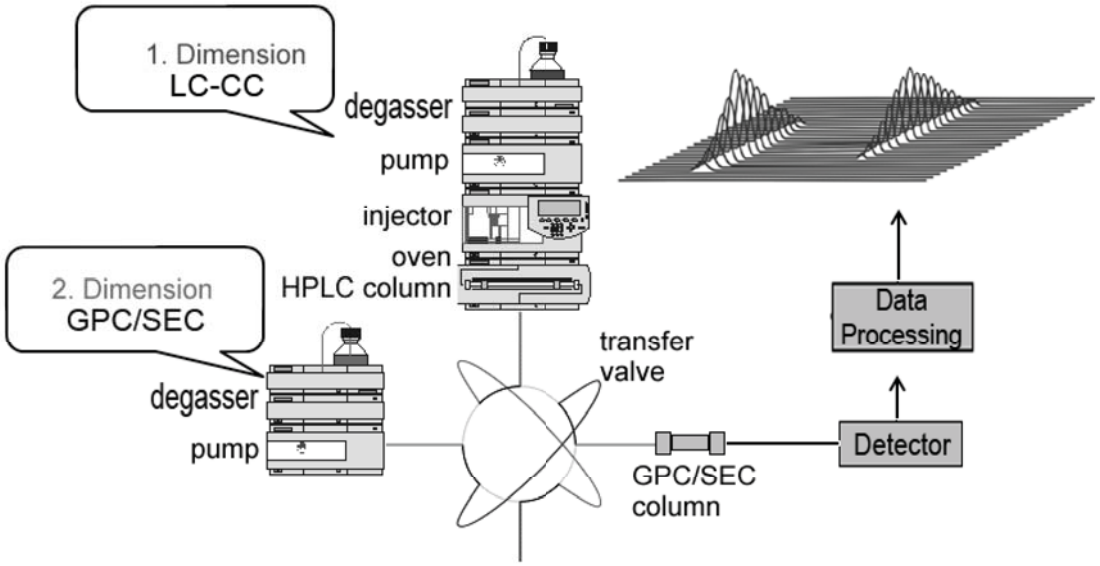


Figure 6.1 Schematic representation of the 2D chromatography setup combining LCCC and SEC techniques.[74]

Throughout the presented thesis, LCCC conditions of PEtOx polymers were identified for the first time in a collaboration study with two other PhD students. The details of the synthesis and the identified LCCC conditions are given in previously published PhD theses; the application of the developed LCCC method was already used to analyze PEtOx containing block copolymers and published in a research article (Publication 9 of this thesis).[75-77] In addition, a PEtOx based comb polymer with a methacrylate backbone was successfully analyzed by the established 2D LCCCxSEC setup. The retention behavior of the comb polymer verified the macromolecular bottle brush structure in solution.[78] Moreover, the integration of an automated spotting robot in 2D chromatography setup which leads to a faster and online analysis procedure was also successfully accomplished in a separate study.[79] The developed 2D method can be employed to analyze a variety of other functional PEtOx based homo- and copolymers enabling the quantification of side-products, which is very important due to the possible bio-medical applications of this polymer in the future. Here, in this chapter, the focus is on the hyphenation of a 2D technique to MS and MS/MS techniques for the complete characterization of PEtOx copolymers.

Subsequent to the separation utilizing the developed 2D setup, three fractions were obtained from the 2D analysis of a P(EtOx-*b*-oDFOx) copolymer (**P29**) (Figure 6.2). The 2D analysis proved the presence of different species other than the desired copolymer structure. The 1st (7.5 vol. %) and the 3rd (4.5 vol. %) fractions consist PEtOx homopolymers and the 2nd fraction contains only the desired block copolymer (87 vol. %). It should be noted that without the LCCC-SEC coupling in a 2D manner, all three fractions would elute at the same time during the standard SEC analysis. This situation underlines on the importance of a 2D analysis for the complete characterization of these copolymers and the quantitative information achieved from this study. All obtained fractions were subsequently characterized by MALDI-TOF MS as well as ESI-Q-TOF MS and MS/MS techniques. The acquired MS data proved that the 1st and the 3rd fractions belong to PEtOx homopolymers with exactly the same *m/z* values and the 2nd fraction belongs to the block copolymer. ESI-Q-TOF MS/MS data allowed the

differentiation of the PEtOx polymers with different end-groups but same *m/z* values due to the special fragmentation behavior of the side product. These PEtOx homopolymers were obtained because of the side reactions occurred during the termination of the CROP process. During the termination process, the termination agent (water in this case) can attack the oxazolinium species at two different positions causing two different end-groups, namely a hydroxyl end-group [$CH_3(C_5H_9NO)_nOH$] or an amino / ester end-group [$CH_3(C_5H_9NO)_{n-1}NHC_2H_4CO_2C_2H_5$]. However, both species have identical *m/z* values and it is not possible to differentiate between them via single-stage MS experiments. Nevertheless, the existence of the side product (with an amino/ester end-group) can be confirmed via tandem MS experiments due to the different fragmentation patterns (PEtOx with the ester end-group was easily dissociated via a 1,5-hydrogen rearrangement reaction; in contrast, PEtOx with the hydroxyl end-group fragmented through backbone cleavages),[37, 50, 77] which makes the application of MS/MS analysis obligatory in order to characterize individual end-groups (Figure 6.2). Therefore, MS/MS analysis is required to distinguish isobaric and isomeric species to determine distinct end-groups, macromolecular connectivities and architectures completely.

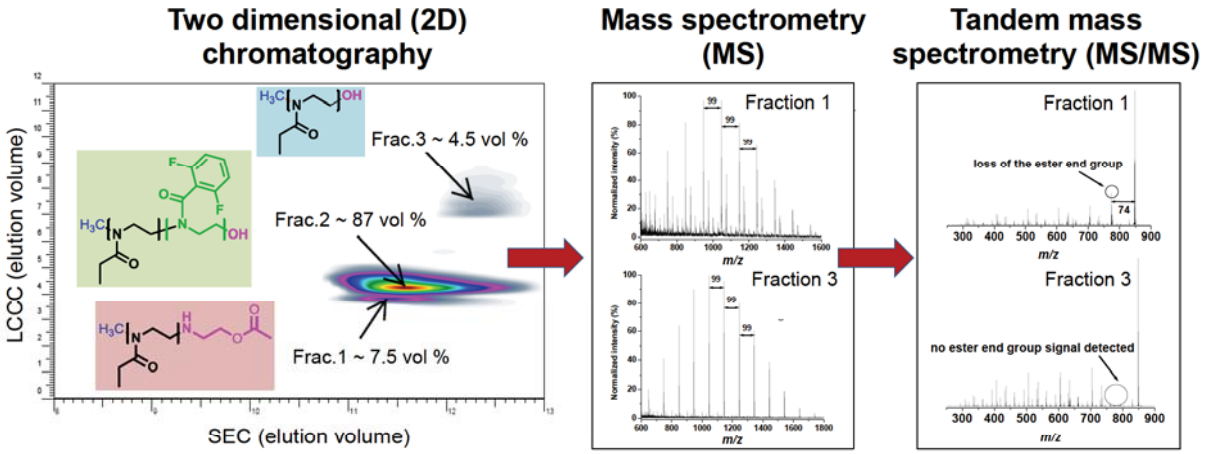


Figure 6.2 The usage of LCCC-SEC (2D) analysis together with ESI-Q-TOF MS and MS/MS for the complete characterization of PEtOx polymers.

The immense advancement of various polymerization techniques in contemporary polymer science has led to complex polymeric systems comprised of a variety of building blocks. In recent years, 2D chromatography and MS-based techniques have separately provided solutions for the polymer science community to analyze these complex polymers. However, the obtained results during this thesis showed that it is necessary to combine these techniques for an efficient characterization. For that reason, the coupling of different chromatographic techniques is required to simplify complex polymer molecules according to their different features (*i.e.* molar mass, functionality, composition, etc.) prior to MS and MS/MS analysis to accomplish a detailed and effective characterization of synthetic polymers (Figure 6.3). Hopefully, in the future the combination of the various chromatographic setups to MS techniques will be available in an automated manner utilizing different on-line spotting and spraying tools for a further improved characterization.

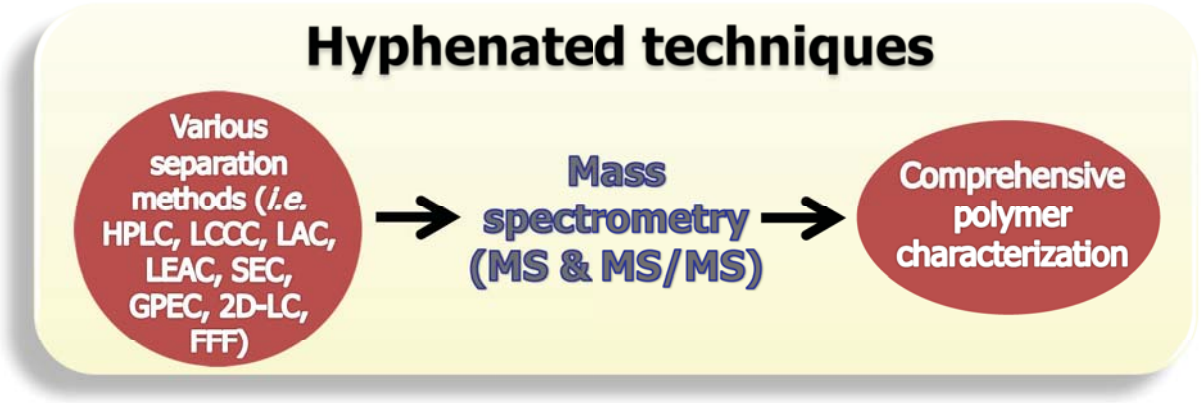


Figure 6.3 Hyphenation of separation methods to MS-based techniques for polymer characterization.

7. Software interpretation towards "Polymeromics"

Parts of this chapter have been published: P5) E. Altuntaş, A. Baumgaertel, A. Krieg, A. C. Crecelius, U. S. Schubert, *J. Polym. Sci., Part A: Polym. Chem.* **2013**, *51*, 1595-1605. P7) E. Altuntaş, K. Kempe, C. Weber, U. S. Schubert, *Eur. Polym. J.* **2013**, *49*, 2172–2185.

As stated in the previous chapters, the analysis of synthetic polymer samples presents special challenges and limitations because of their complex structure. MS-based techniques together with appropriate separation techniques can provide solutions for the polymer science community. MS/MS analysis of synthetic polymers is particularly attractive, because other techniques cannot provide such detailed structural information by allowing comprehensive sequence information to be derived from the mass spectra. Currently, possessing expert knowledge and certain skills are required for the manual interpretation of the MS/MS data obtained from synthetic polymers. On the other hand, the utilization of MS-based strategies in polymer science has recently become routine. As a result, the number of novice users of these strategies has increased. The major difficulty at this point is the transformation of tandem mass spectral data of synthetic polymers into chemically valuable information with a minimum of required involvement of the user, preferably through automated interpretation of the complex (co)polymer fragmentation patterns. The advances in software development and the large availability of open source software solutions for complicated MS/MS data in OMICS sciences have greatly assisted many scientists in various research fields, especially in proteomics.[30, 32, 34, 80] In a similar manner, polymer researchers can also benefit from the development of such software tools for the analysis of polymers via MS/MS. Therefore, in order to achieve insights of the macromolecular structure with MS-based methods, it is important to develop special data interpretation software tools to identify fragmentation products obtained by MS/MS as in the fields of various OMICS sciences. Even though the extreme importance of computational analysis of MS/MS data from polymers, it is unfortunately still an embryonic field of research and this is accepted to be one of the major problems in the MS/MS analysis of polymers. Before the development of the PLUMS (Polymer labeling using mass spectrometry) software by Boecker *et al.*, [81] there was only one software tool available for the evaluation of polymer MS/MS data developed by Thalassinos *et al.* (Polymerator software).[82] Thalassinos and coworkers have applied this software to analyze various polymer classes such as polymethacrylates, polyethers, and polystyrenes.[82-86] The Polymerator software allows polymer scientists to validate the proposed structures to be verified by automatically interpreting the data file by previously suggested fragmentation rules for synthetic polymers. The utilization of the software enables a fast annotation of the MS/MS data. However, this software requires the knowledge of the fragmentations pathways of the investigated polymers and information about the main repeating unit, the number of the main repeating units, the initiating group (α), the terminating group (ω) and the cationization agent. The PLUMS software tool has been developed in a collaboration between the Boecker group (Department of Bioinformatics, FSU Jena) and the Schubert group to accelerate the interpretation of MS/MS spectra without requiring any further knowledge about the polymer class or the fragmentation behavior. This software simply

requires an alphabetical list of elements and a peak list of the measured substance as a XML file for the assessment of the selected mass spectrum. The software determines the monomer repeating unit and the formulae for the corresponding end groups of the series of fragmentation products.

In this part of the thesis, the application of this special software tool to evaluate MS/MS data from synthetic polymers is discussed. This software was originally developed for MALDI MS/MS data; therefore, it has already been utilized to analyze MALDI MS/MS data of homopolymers. Moreover, the application of this data interpretation software to ESI and APCI MS/MS data was successfully reported as well.

The usage of the PLUMS software for the interpretation of PMA polymer (**P15**) is shown in Figure 7.1. The findings suggest that this software tool represents an important help in accomplishing the interpretation of tandem MS spectra of homopolymers in a fast and automated manner. Applying a mass error of 0.5 *m/z* units for the MALDI-TOF MS/MS data, the software identifies five different fragment series with an appropriate end-group formula. Comparing the manual and the automated evaluation output, it is clearly visible that both interpretation methods delivered the same results.

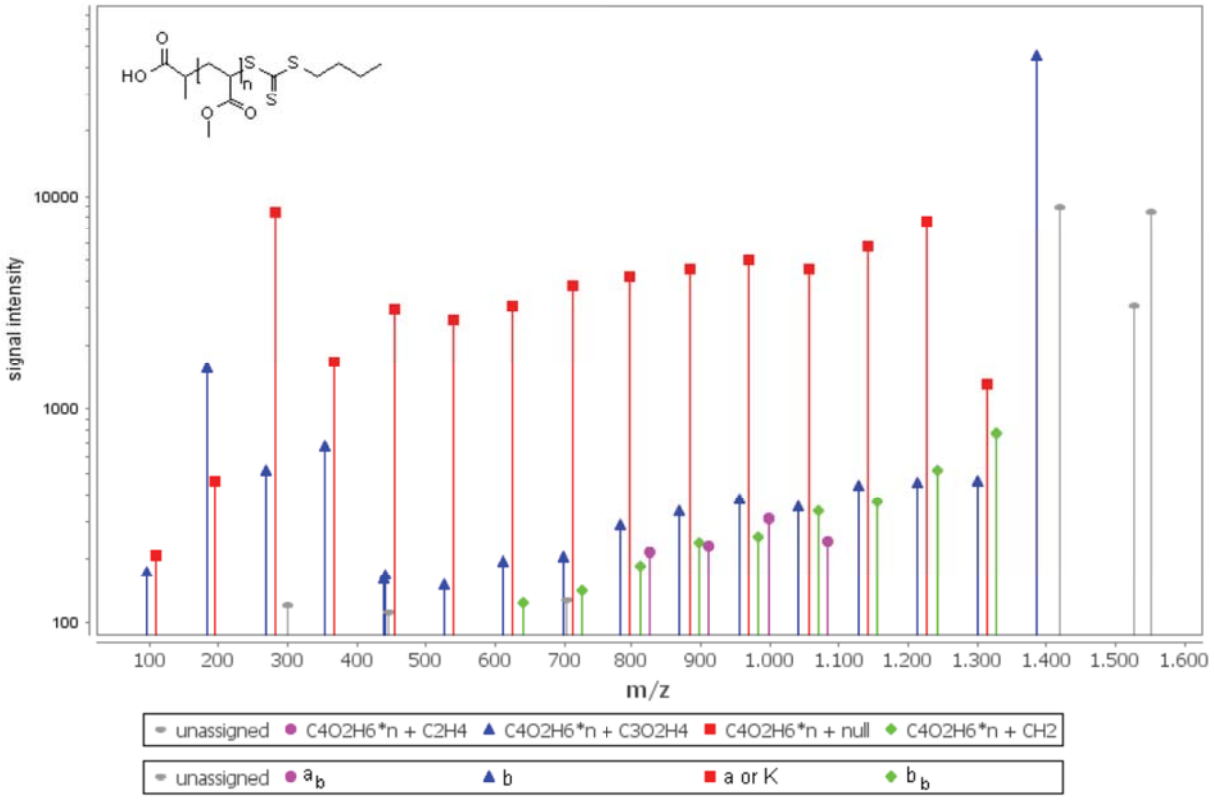


Figure 7.1 MALDI-TOF MS/MS spectrum of PMA homopolymer (**P15**): Evaluation by the new PLUMS software.

The evaluation of ESI and APCI MS/MS spectra of the PEtOx polymer (**P14**) is depicted in Figure 7.2. The software provided a value for the repeating unit of *m/z* 99.07 within different fragmentation series. The calculated value fits exactly to the mass of the EtOx repeating unit (C_5H_9NO). The

automatic interpretation delivered several fragmentation series, which are ascribed to the depolymerization mechanism and were already assigned manually.

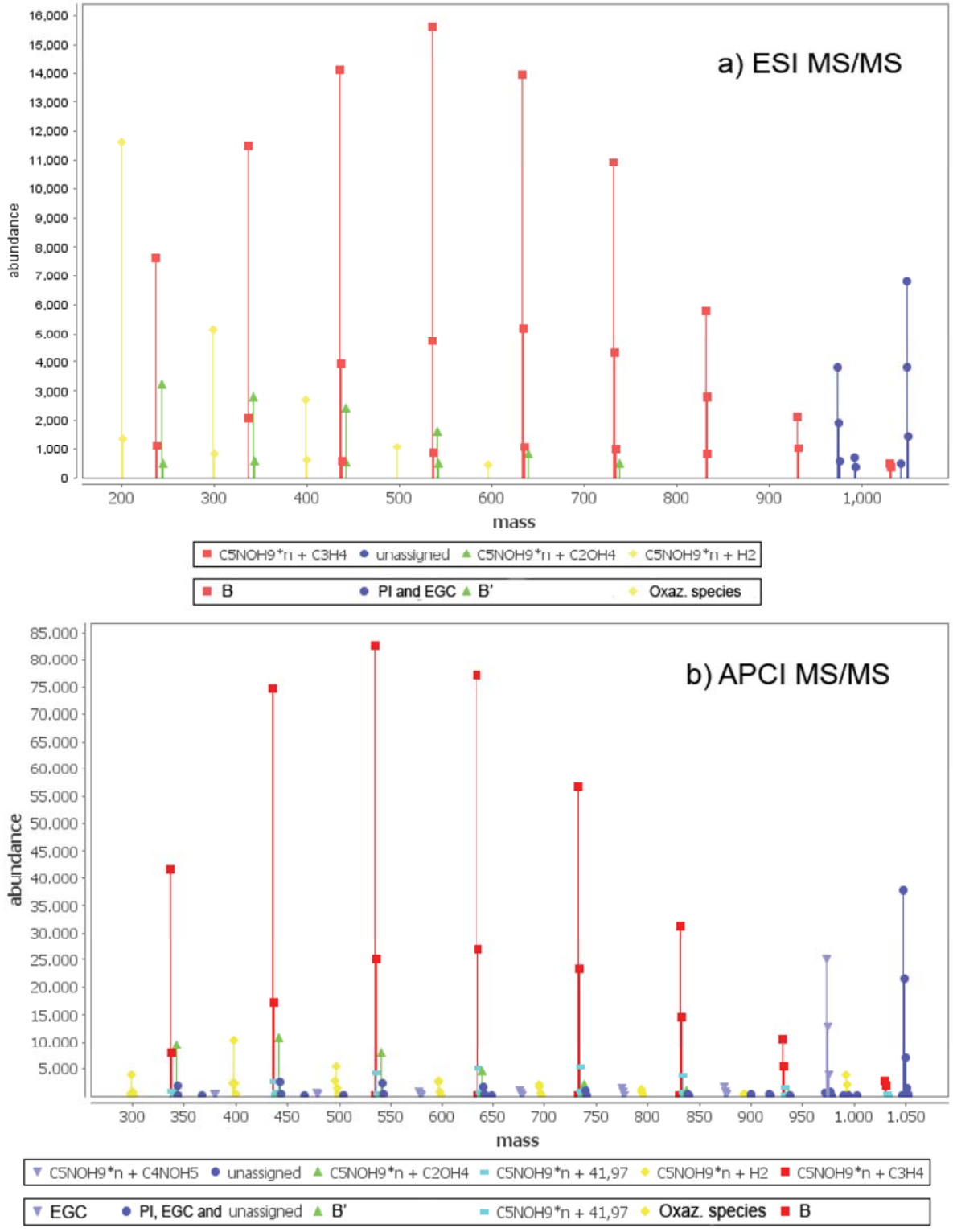


Figure 7.2 ESI and APCI MS/MS spectra of PEtOx (**P14**): Evaluation by the PLUMS software (EGC: End-group cleavage, PI: Precursor ion).

All in all, it can be concluded that creating tandem mass spectral libraries for synthetic polymers is the most important task on the way to "Polymeromics". For that reason, defining the characteristic fragmentation patterns for a certain type of synthetic polymer is important and this information can be used as a reference to characterize unidentified polymers within the same class. To accomplish this purpose, the PLUMS software enables polymer scientists to achieve straightforward identification of fragmentation patterns for synthetic polymers without laborious manual interpretation efforts (Figure 7.3).

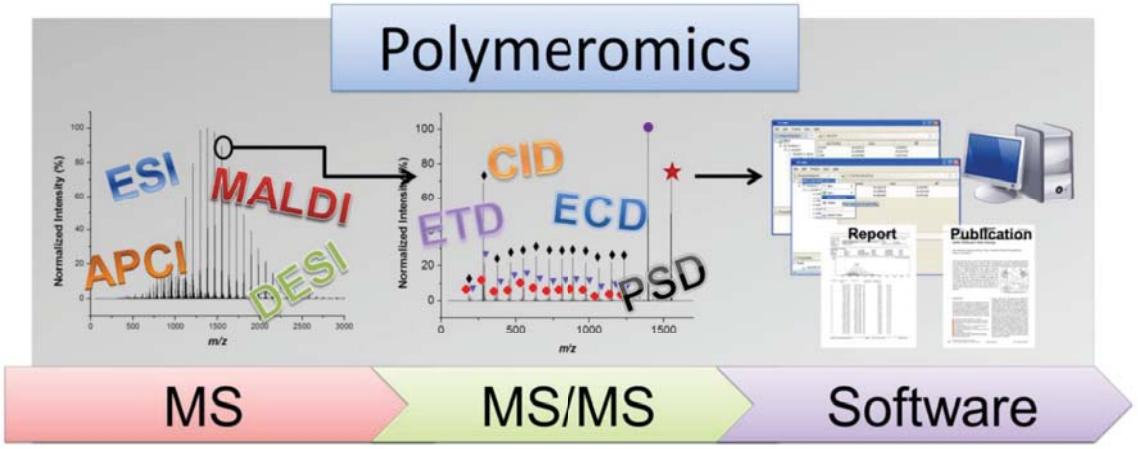


Figure 7.3 MS-based strategies in polymer science and characterization towards "Polymeromics".

The preliminary results obtained up to now are certainly fascinating and open some interesting perspectives for the future developments of software tools. As expected, the future challenge will be the developments of new software tools to analyze MS/MS data from more complex systems such as various polymers with different architectures (*i.e.* branched and cyclic polymers), copolymers, and star-shaped polymers. However, the developments of new software tools are not sufficient to achieve the aim of polymer sequencing. As mentioned earlier, it is necessary to build reference libraries for fragmentation product ions of polymers and to provide open-source MS and MS/MS data for all polymer scientists. In order to achieve this goal, Deutsch and co-workers introduced the 'mzXML' format, an open, generic XML output of MS data. Moreover, the authors have also developed an accompanying suite of supporting programs. The expectation from this format is to facilitate data management, interpretation and distribution in proteomics research.[87-91] A similar approach can be utilized in polymer research via MS-based techniques (MS, MS/MS or MS^n) in order to form a common (open source) data analysis framework for the polymer community (Figure 7.4). This framework will embrace the need for a publicly accessible database of polymers analyzed via MS-based studies as well as software tools that provides the polymer community a platform to select and validate mass spectrometry strategies.

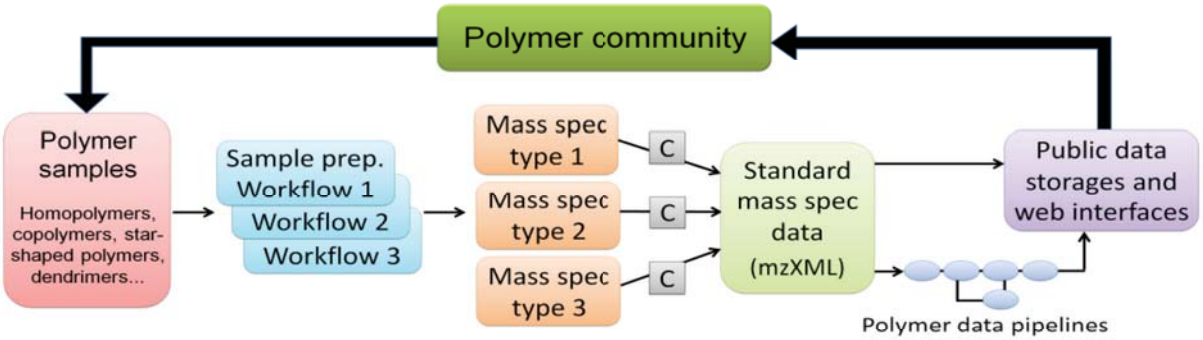


Figure 7.4 Open source data analysis framework for "Polymeromics". The mzXML file acts as a mediator, allowing multiple input formats to be subjected to a common data analysis pipeline. New types of instruments can be integrated into a preexisting analysis framework with only a utility (here represented by C) to convert MS native output to the mzXML format. The open structure of mzXML instance documents makes them suitable for data exchange, for example, they may be submitted to a data repository to support the results presented in a publication or a report (modified based on similar approaches).[91, 92]

In summary, the application of a special software tool for the evaluation of polymer MS/MS data was successfully shown in this part of the thesis. The PLUMS software was effectively utilized not only for the elucidation of MALDI MS/MS data, but also for ESI and APCI MS/MS data. The research groups of Boecker and Schubert are currently working on a new software tool focused on the expansion of this software including the interpretation of MS/MS spectra from block and random copolymers with a sequence analysis (*de novo* sequencing for polymers).

8. Summary

The objective of the presented thesis was to investigate the applicability of mass spectrometry (MS)-based techniques for the analysis of polymers and to highlight their great potential for the detailed characterization of a wide range of synthetic polymers. A variety of MS and tandem MS (MS/MS) techniques were utilized to gain accurate structural information from intricate macromolecular structures. Up to now, a wide range of successful studies were published reporting the usage of matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) for polymer analysis. In this work, electrospray ionization mass spectrometry (ESI MS) and atmospheric pressure chemical ionization mass spectrometry (APCI MS) were heavily utilized to gain structural information of synthetic polymers (Chapter 2). In particular, the ESI MS technique was shown to be a distinguished soft ionization technique for the characterization of synthetic polymers with fragile end-groups as well as supramolecular compounds. The potential of this technique was proven by different studies presented in this thesis.

A wide range of synthetic polymers (*i.e.* POx, PEI, PMA, etc.) were comprehensively analyzed by ESI, APCI and MALDI MS in systematic comparison studies. In general, the ESI and APCI methods were found to be comparable with respect to end-group determination capabilities and both techniques generally resulted in less in-source fragmentation compared to the MALDI technique for the investigated polymers. However, the MALDI technique was found to be superior regarding molar mass determination. The obtained results of the systematic comparison studies were summarized in Chapter 3.

Several polymer classes were investigated via detailed MS/MS experiments (Chapter 4). The complementary structural information was achieved using MS/MS analysis to identify distinct end-group functionalities, to distinguish isobaric and isomeric species and to analyze comprehensively the macromolecular architectures of various polymers. Additional to these applications of MS/MS, characteristic fragmentation patterns have been defined for several polymer classes and the effect of end-groups on the fragmentation mechanisms were scrutinized. The ultimate goal of these studies was to create tandem MS product ion libraries providing the necessary data for a fast and automated identification of these polymers in the future.

Additionally, MS/MS studies were further extended and the energy-dependent collision induced dissociation (CID) experiments were performed in order to investigate the dependency of the fragmentation patterns of synthetic polymers as a function of collision energy (Chapter 5). The survival yield (SY) method was used to determine the characteristic collision energy (CE_{50}) values to gain a complete picture of fragmentation mechanisms and stabilities of polymers as well as of supramolecular compounds. In these studies, SY curves were shown as a useful tool to correlate the macromolecular structure of poly(2-ethyl-2-oxazoline)s (PEtOxs) to their fragmentation behavior. Moreover, energy dependent CID experiments were employed to analyze the gas-phase stabilities of various metal complexes in this part of the thesis.

In Chapter 6, the hyphenated techniques for polymer characterization were discussed in detail for the case of PEtOx based copolymers. LCCC conditions were identified for PEtOx polymers and the 2D setup employing LCCC-SEC coupling combined with ESI MS/MS was utilized to accurately separate and verify the PEtOx containing copolymer as well as its side products. In this study, the necessity to combine different chromatographic techniques to simplify complex polymer samples prior to MS or MS/MS analysis was emphasized in order to accomplish a detailed characterization of the polymers.

In Chapter 7, the application of a special software tool (PLUMS) was discussed to automatically evaluate MS/MS data from synthetic polymers. The findings suggest that this software tool is an important assistant in achieving a fast and automated interpretation of tandem MS spectra of homopolymers like in other OMICS sciences. On the way to "Polymeromics" (Figure 8.1), software tools like PLUMS will enable polymer scientists to achieve a straightforward identification of the fragmentation patterns of synthetic polymers without laborious manual interpretation efforts.

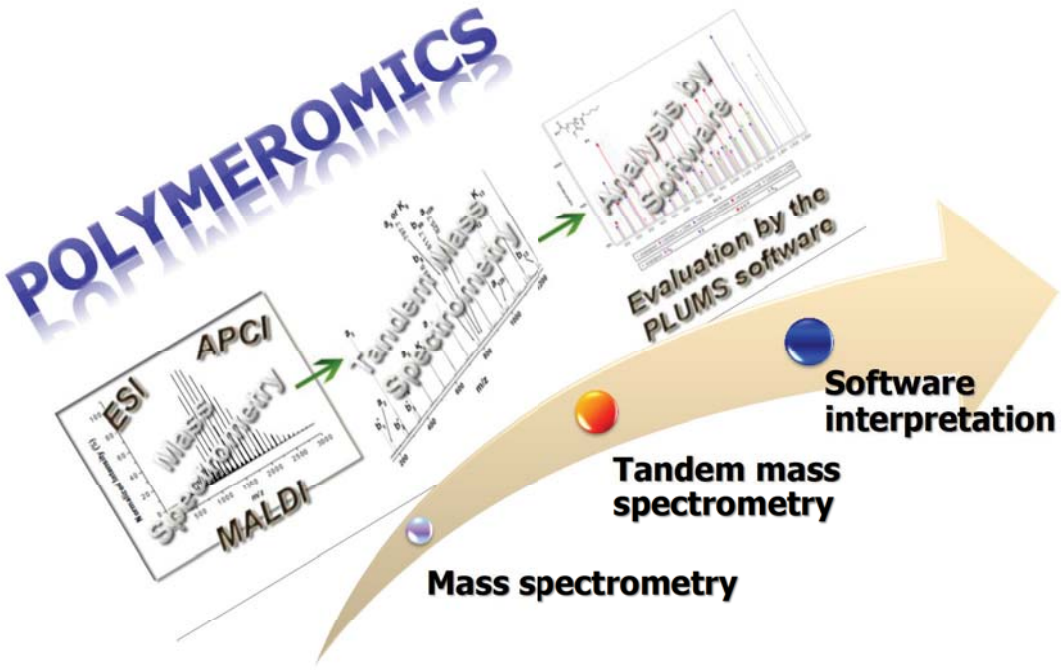


Figure 8.1 Schematic representation of the "Polymeromics" concept introduced in this thesis.

To sum up, the work described in this thesis presents the powerful utilization of MS-based techniques for the characterization of various synthetic polymers. In combination with proper separation techniques and data analysis software tools, unique structural information can be obtained via MS-based techniques. Future work will focus on developing new software tools for more complex systems like block and random copolymers to reach the ultimate goal of polymer sequencing. Moreover, it is crucial to construct reference libraries for fragmentation product ions of polymers and to provide open-source MS and MS/MS data for polymer scientists like in other OMICS sciences.

9. Zusammenfassung

Das Ziel der vorgestellten Arbeit war es zu untersuchen, inwieweit massenspektrometrische Techniken für die Analyse von Polymeren anwendbar sind. Um das große Potenzial für die detaillierte Charakterisierung von einer Reihe von synthetischen Polymeren zu verdeutlichen, wurden verschiedene MS und Tandem-MS Techniken angewendet, die es erlauben, genaue strukturelle Informationen über komplizierte makromolekulare Strukturen zu erhalten. Bisher wurden eine Vielzahl von Studien über die erfolgreiche Polymeranalyse mittels Matrix-unterstützter Laser-Desorptions-Ionisierungs Massenspektrometrie (MALDI MS) veröffentlicht. In dieser Arbeit wurde diese Palette durch die verstärkte Hinzuziehung von Elektrospray-Ionisierungs Massenspektrometrie (ESI MS) und Atmosphärendruck chemischer Ionisierungs-Massenspektrometrie (APCI MS) erweitert, um strukturelle Informationen über synthetische Polymere zu erhalten (Kapitel 2). Insbesondere ESI MS zeichnete sich dabei als hervorragende weiche Ionisierungstechnik für die Analyse von synthetischen Polymeren mit fragilen Endgruppen sowie supramolekularen Verbindungen aus. Das Potenzial dieser Technik wird durch die verschiedenen Studien, die in dieser Arbeit vorgestellt werden, bewiesen.

Eine Vielzahl von synthetischen Polymeren (d. h. POx, PEI, PMA, usw.) wurde umfassend durch ESI, APCI und MALDI MS in systematischen Vergleichsstudien analysiert. Im Allgemeinen lieferten ESI und APCI vergleichbare Ergebnisse bezüglich der Endgruppen-Bestimmung, und beide Techniken führten, im Vergleich zu MALDI, zu weniger Fragmentierung in der Quelle für die untersuchten Polymere. Andererseits stellt MALDI eine bessere Technik für die Bestimmung von molaren Massen dar. Die erzielten Ergebnisse der systematischen Vergleichsstudien sind in Kapitel 3 zusammengefasst.

Mehrere Polymerklassen wurden mittels detaillierter MS/MS Experimente untersucht (Kapitel 4). Hierbei wurde ergänzende strukturelle Informationen gewonnen, um bestimmte funktionelle Endgruppen zu identifizieren, zwischen isobaren und isomeren Spezies zu unterscheiden und eine allumfassende Analyse der makromolekularen Architekturen verschiedener Polymere durchzuführen. Zusätzlich wurden charakteristische Fragmentierungsmuster für einige Polymerklassen definiert, und der Einfluss der Endgruppen auf die Fragmentierungsmechanismen erforscht. Das letztendliche Ziel dieser Studien besteht darin, Bibliotheken von MS Produktionen zu generieren, die die benötigten Daten für eine zukünftige schnelle und automatisierte Identifikation dieser Polymere liefern.

Des Weiteren wurden die MS/MS Studien durch Experimente mit energieabhängiger kollisionsinduzierter Dissoziation (CID) ergänzt, um die Fragmentierungsmuster hinsichtlich ihrer Abhängigkeit auf die Kollisionsenergie zu untersuchen (Kapitel 5). Hierfür wurde die Überlebensausbeute (engl. Survival yield, SY) Methode angewendet, um charakteristische Kollisionsenergie-Werte (CE_{50}) zu bestimmen und so das erhaltene Bild (Fragmentierungs-mechanismen und Stabilitäten) von Polymeren und supramolekularen Verbindungen zu komplettieren. Durch diese Studien konnte gezeigt werden, wie nützlich SY Kurven sind, um die makromolekulare Struktur von PEtOxs mit dem Fragmentierungsverhalten zu korrelieren. Zusätzlich wurden die energieabhängigen CID Experimente genutzt, um Stabilitäten von verschiedenen Metallkomplexen in der Gasphase zu ermitteln.

In Kapitel 6 wurden kombinierte Techniken für die Charakterisierung von Polymeren im Detail für PEtOx-basierte Copolymere erläutert. Die kritischen Bedingungen für PEtOx wurden identifiziert und durch eine Kombination von 2D LCCC-SEC Kopplung und ESI MS/MS konnten alle Bestandteile der Probe (das Copolymer sowie die Nebenprodukte) getrennt und deren Strukturen verifiziert werden. Durch diese Studie konnte gezeigt werden, wie wichtig es ist, die Analyse von MS oder MS/MS bei Proben mit komplexer Zusammensetzung durch Kombination mit geeigneten chromatographischen Techniken zu ergänzen, um eine detaillierte Charakterisierung der Polymere zu gewährleisten.

In Kapitel 7 wurde die Anwendung einer speziellen Software (PLUMS) zur automatischen Auswertung von MS/MS Spektren synthetischer Polymere diskutiert. Die Resultate zeigen, dass diese Software ein wichtiges Hilfsmittel für die schnelle und automatische Interpretierung von tandem MS Spektren von Homopolymeren darstellt, wie in anderen "OMICS" Wissenschaften. Auf dem Weg zu "Polymeromics" (Abbildung 8.1) werden Software-Anwendungen wie PLUMS den Polymer-wissenschaftlern dabei helfen, Fragmentierungsmuster von synthetischen Polymeren unkompliziert und ohne aufwändige manuelle Interpretation zu identifizieren.

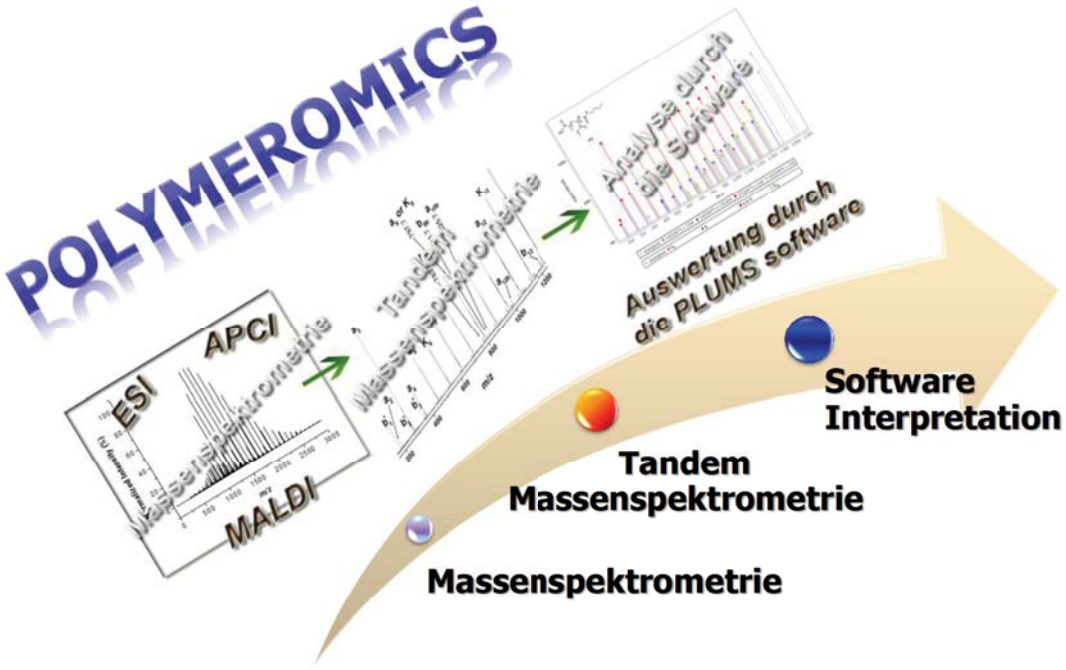


Abbildung 8.1. Schematische Darstellung des vorgestellten "Polymeromics" Konzeptes.

Zusammenfassend konnte in dieser Arbeit die Leistungsfähigkeit MS-basierter Techniken für die Charakterisierung von unterschiedlichen synthetischen Polymeren demonstriert werden. In Kombination mit Trenntechniken und Software-Hilfsmitteln zur Datenanalysierung kann einzigartige strukturelle Information durch MS-basierte Techniken gewonnen werden. In der Zukunft soll die Software weiterentwickelt werden, so dass diese auch auf komplexere Systeme wie Block- oder Randomcopolymere anwendbar ist, um das ultimative Ziel "Polymer-Sequenzierung" zu erreichen. Außerdem ist es unumgänglich, Referenz-Bibliotheken für Fragmentionen-Produkte von Polymeren als Basis für "open-source" MS und MS/MS Datenbanken zu realisieren, auf die die Polymerwissenschaftler uneingeschränkten Zugang haben, wie in den anderen "OMICS"-Wissenschaften.

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List of abbreviations

2D-LC	two-dimensional liquid chromatography
AIBN	2,2'-azobis(isobutyronitrile)
APCI	atmospheric pressure chemical ionization
ATRP	atom transfer radical polymerization
CAP	critical point of adsorption
CCD	chemical composition distribution
CCE or CE_{50}	characteristic collision energy
CID	collision-induced dissociation
CROP	cationic ring-opening polymerization
CRP	controlled radical polymerization
Da	Dalton (molar mass unit)
DCM	dichloromethane
DCTB	*trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile
DHB	2,5-dihydroxy benzoic acid
Dithranol	1,8,9-anthracenetriol
ELSD	evaporative light scattering detector
ESI	electrospray ionization
FTD	functionality type distribution
$g \cdot mol^{-1}$	grams per mole
GPEC	gradient polymer elution chromatography
HPLC	high-performance liquid chromatography
L/CRP	living / controlled radical polymerization
LAC	liquid adsorption chromatography
LC	liquid chromatography
LCCC	liquid chromatography at critical conditions
m/z	mass-to-charge ratio
MAD	molecular architecture distribution
MALDI	matrix-assisted laser desorption/ionization
MMD	molar mass distribution
M_n	number average molar mass
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	tandem mass spectrometry
M_w	weight average molar mass
mzXML	an open data format for storage and exchange of MS data
NMP	nitroxide mediated polymerization
NMR	nuclear magnetic resonance
PCIS	precursor ion selector

PDI	polydispersity index
PEI	poly(ethyleneimine)
PEPOx	poly(2-(1-ethyl-pentyl)-2-oxazoline)
PEtOx	poly(2-ethyl-2-oxazoline)
PMA	poly(methylacrylate)
PMetOx	poly(2-methyl-2-oxazoline)
P*o*DFOx	poly(2-*o*-difluoro-2-oxazoline)
POx	poly(2-oxazoline)
PPhenOx	poly(2-phenyl-2-oxazoline)
P*p*TBPhOx	poly(2-*p*-*ter*butylphenyl-2-oxazoline)
Q-TOF	quadrupole time-of-flight
RAFT	radical addition fragmentation chain transfer
ROP	ring-opening polymerization
SEC	size-exclusion chromatography
SY	survival yield
TOF	time-of-flight
TPIC	temperature gradient interaction chromatography
XML	extensible markup language
α	initiating end-group
ω	terminating end-group

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12.09.1983	Born in Istanbul, Turkey
1989-2000	Primary school and high school education in Istanbul, Turkey
2000-2005	Bachelor of Science (B.Sc.) degree at the Hacettepe University (Ankara, Turkey) with graduation work under the supervision of Prof. Dr. Murat Sen and Prof. Dr. Olgun Güven
2002-2004	Part-time job at the Material and Chemistry laboratories (Hacettepe University, Ankara, Turkey)
2004	Internship at Akzo Nobel Chemicals, Marshall Paint Industry (2 months)
2004	Research student at the Sabanci University (Kocaeli, Turkey) in the group of Prof. Dr. Yuda Yürüm (2 months)
2005-2008	Master of Science (M.Sc.) degree at the Boğaziçi University (Istanbul, Turkey) under the supervision of Prof. Dr. Nihan Nugay
2007-2008	Junior Scientist Scholarship, The Scientific and Technological Research Council of Turkey (TUBITAK).
01/2008-11/2008	R&D manager in Bostik-Turkey (The Adhesive Company)
2009-2013	PhD student and teaching assistant at the Laboratory of Organic and Macromolecular Chemistry (IOMC) at the Friedrich Schiller University Jena under the supervision of Prof. Dr. Ulrich S. Schubert
2009	Short scientific visit to the BAM for 2D chromatography studies (Bundesanstalt für Materialforschung und –prüfung) (Berlin, Germany)
2009	Short scientific visit to the Karlsruhe Institute of Technology (group of Prof. Dr. Christopher Barner-Kowollik, Karlsruhe, Germany)

2011	Best poster award at the IUPAC 9th International Conference on Advanced Polymers via Macromolecular Engineering (APME 2011), Cappadocia, Turkey.
2013	Best poster award at the 6th International Symposium on the Separation and Characterization of Natural and Synthetic Macromolecules (SCM-6), Dresden, Germany.
2013	Travel scholarship from the FSU Jena ProChance program in order to participate German Society of Mass Spectrometry conference (DGMS).

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Declaration of authorship / Selbstständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

I certify that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigations, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other university.

Jena, _____ _____

Esra Altuntaş

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Publication P1:

"Polymeromics": Mass spectrometry (MS) based strategies in polymer science towards complete sequencing approaches

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Esra Altuntaş, and Ulrich S. Schubert

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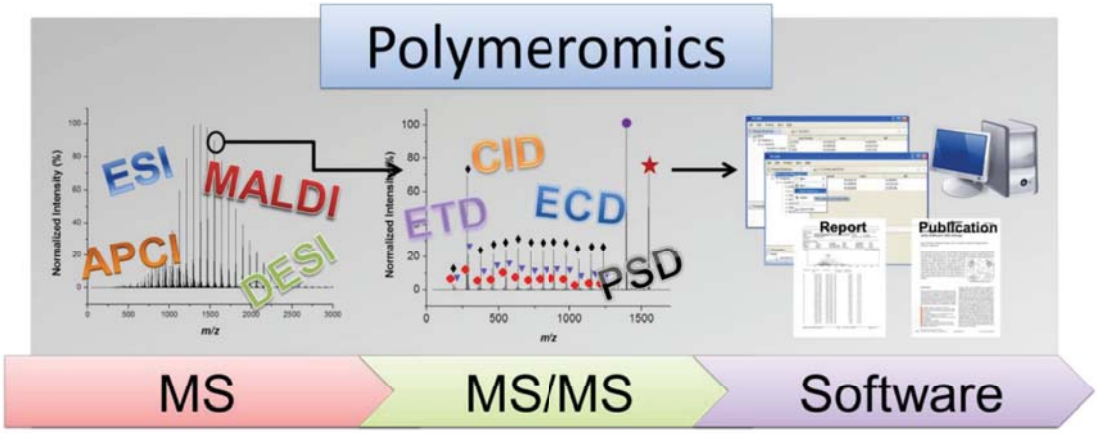

"Polymeromics": Mass spectrometry (MS) based strategies in polymer science towards complete sequencing approaches

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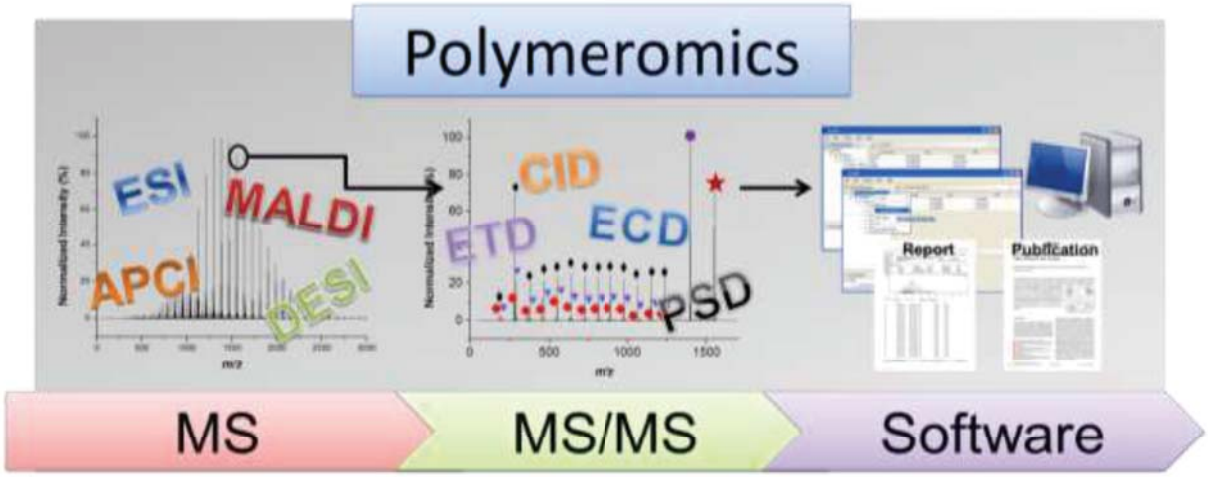
ABSTRACT

Mass spectrometry (MS) is the most versatile and comprehensive method in "OMICS" sciences (*i.e.* in proteomics, genomics, metabolomics and lipidomics). The applications of MS and tandem MS (MS/MS or MS^n) provide sequence information of the full complement of biological samples in order to understand the importance of the sequences on their precise and specific functions. Nowadays, the control of polymer sequences and their accurate characterization is one of the significant challenges of current polymer science. Therefore, a similar approach can be very beneficial for characterizing and understanding the complex structures of synthetic macromolecules. MS-based strategies allow a relatively precise examination of polymeric structures (*e.g.* their molar mass distributions, monomer units, side chain substituents, end-group functionalities, and copolymer compositions). Moreover, tandem MS offer accurate structural information from intricate macromolecular structures; however, it produces vast amount of data to interpret. In "OMICS" sciences, the software application to interpret the obtained data has developed satisfyingly (*e.g.* in proteomics), because it is not possible to handle the amount of data acquired *via* (tandem) MS studies on the biological samples manually. It can be expected that special software tools will improve the interpretation of (tandem) MS output from the investigations of synthetic polymers as well. Eventually, the MS/MS field will also open up for polymer scientists who are not MS-specialists. In this review, we dissect the overall framework of the MS and MS/MS analysis of synthetic polymers into its key components. We discuss the fundamentals of polymer analyses as well as recent advances in the areas of tandem mass spectrometry, software developments, and the overall future perspectives on the way to polymer sequencing, one of the last Holy Grail in polymer science.

GRAPHICAL ABSTRACT



HIGHLIGHTS

- Publications on MS-based strategies towards sequential analysis of polymers are reviewed.
- New approaches in MS/MS characterization of polymers are discussed.
- Software tools for the automated interpretation of polymer MS data are encouraged.
- Future prospects using MS-based strategies on polymer analysis are suggested.

Abbreviations

2D-LC, two-dimensional liquid chromatography; AUC, analytical ultracentrifugation; APCI, atmospheric pressure chemical ionization; CCE, Characteristic collision energy; CID, collision-induced dissociation; CRP, controlled radical polymerization; ECD, electron capture dissociation; ESI, electrospray ionization; FTMS, Fourier transform mass spectrometry; FTICR, Fourier transform ion cyclotron resonance; GMA, glycidyl methacrylate; GPEC, gradient polymer elution chromatography; HPLC, high-performance liquid chromatography; IMS or IMMS, ion mobility (mass) spectrometry; LAC, liquid adsorption chromatography; LEAC, liquid exclusion adsorption chromatography; LC, liquid chromatography; LCCC, liquid chromatography at critical conditions; LS, light scattering; MALDI, matrix-assisted laser desorption/ionization; MMA, methyl methacrylate; MMD, molar-mass distribution; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; ODNs, oligodeoxynucleotides; PEI, poly(ethylene imine); PCL, poly(ε-caprolactone); PDI, polydispersity index; PDMS, poly(dimethylsiloxane); PE, poly(ethylene); PEG, poly(ethylene glycol); PEO, poly(ethylene oxide); PET, poly(ethylene terephthalate); PEtOx, poly(2-ethyl-2-oxazoline); PEPOx, poly(2-(1-ethylpentyl)-2-oxazoline); PLUMS, Polymer labeling using mass spectrometry; PMAA, poly(methacrylic acid); PMA, poly(methacrylate); PMMA, poly(methyl methacrylate); POx, poly(2-oxazoline)s; PP, poly(propylene); PPO, poly(propylene oxide); PS, poly(styrene); SEC, size-exclusion chromatography; UV-vis, ultraviolet-visible spectroscopy.

Contents

Esra Altuntaş was born in 1983 in Istanbul (Turkey) and studied chemistry at the Hacettepe University (Ankara, Turkey). She completed her M.Sc. degree in 2008 at the Boğaziçi University (Istanbul, Turkey) where she investigated the synthesis and characterization of different polymeric nanocomposites. She worked as a R&D manager for one year at the Bostik Company (Istanbul, Turkey). Currently, she is a PhD candidate at the Friedrich Schiller University Jena (Germany) under the supervision of Prof. Ulrich S. Schubert. Her research focuses on the detailed characterization of synthetic polymers by utilizing various techniques such as (tandem) mass spectrometry (ESI-, MALDI-, and APCI-MS & MS/MS), two-dimensional liquid chromatography (2D-LC), and asymmetrical flow field-flow-fractionation (AF4).

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1. INTRODUCTION

OMICS-sciences have a significant impact on research strategies in life sciences, particularly in molecular biology. Several OMICS-terms were created (*e.g.* glycomics, lipidomics, foodomics, phosphoproteomics, degradomics, toponomics, and interactomics) in the last few years due to the development of high-throughput approaches for the identification, characterization, and quantitation of thousands of biomacromolecules in various research fields. In OMICS-sciences, mass spectrometry (MS) is the most versatile and comprehensive method such as in proteomics, genomics, metabolomics, lipidomics, and foodomics. [1-6] MS represents a highly powerful tool for the identification and quantification of these biomacromolecules and is complementary to other technologies and analysis methods. The applications of MS and tandem MS (MS/MS or MS^n) provide sequence information of the full complement of proteins, genes, metabolites and lipids in biological samples in order to elucidate the influence of the sequences on their precise and specific functions. In many OMICS-sciences, MS-based strategies help researchers to understand diseases on the molecular level and encourage the search for novel and more specific biomarkers for the early diagnosis of diseases, their development and prognosis, as well as the monitoring of their treatments. Application of MS in biological sciences has gained a strong development in the past two decades owing to important developments in experimental methods, instrumentation, and data analysis approaches in these fields. In a similar manner, polymer science and engineering have recently started to appreciate the advancements in the MS-based characterization tools. MS-based strategies have been successfully utilized as advanced characterization methods for synthetic polymers in order to obtain absolute molar mass distributions (MMD), to analyze polymer repeating units, macromolecular architectures, side chain substituents, end-group functionalities and to scrutinize copolymer composition, degradation products, polymerization mechanisms and kinetics. [7-11]

In contemporary polymer science, controlling the sequence of building blocks in polymers offers new opportunities for tailoring the properties of many synthetic polymers for potential applications in different fields such as in chemistry, physics, material science as well as in biological and medical sciences. [12-16] Sequence-defined biomacromolecules (*i.e.* proteins, genes, metabolites and lipids) are fundamental components of the living world, and sequence-controlled synthetic macromolecules will be the most important components of future's material design, particularly in the design of pharmaceutically significant polymers such as polymer therapeutics, polymer-drug conjugates, polymer-protein conjugates, polymeric micelles, and multicomponent polyplexes. Research on these polymers has led to success over the past few decades in mediating safe and effective delivery of bioactive agents to treat a variety of medical conditions. Such systems

are based on a large variety of linear and branched (co)polymers or cross-linked polymer networks and are commercially available with different molar masses and copolymer compositions. Their degradation time can vary from several months to several years depending on the molar masses and copolymer ratio. In order to become marketable products, the mechanical properties and the rates of biodegradation of these polymers have to be investigated in order to receive approval from institutions like the US Food and Drug Administration Agency (FDA) and the European Medicine Agency (EMA). These properties could be adjusted using modern living and controlled polymerization strategies; therefore, the ability to characterize the accurate copolymer sequences has an immense impact in this field. The importance of connecting the molecular structure for understanding properties and the necessity of fast analysis for quality and regulatory assurance has directed to a search for different means of analyzing polymeric structures. We believe that MS-based strategies can contribute to the sequential analysis of these synthetic polymers, by allowing a relative precise examination of the molecular architectures of polymers. These sophisticated characterization tools can help to investigate structure-property relationships and to understand how polymers and polymer products interact with biological systems. Besides, sequence-controlled synthetic polymers are not only important for medical or drug delivery applications but also for other copolymer applications in different fields such as in photonics, solar cells, blend compatibilizers, nanolithography, self-assemblies, stimuli responsive materials, membranes and nanostructures. [17] Therefore, the sequence analysis of these polymers is an important issue in many different research fields and MS-based strategies represent novel methods for the determination of the molecular structure of these polymers. The recently defined "Polymeromics" field that studies polymeric structures through the application of MS-based techniques is of great essence because the challenges for further development of sequence-controlled macromolecular design depends not only on developing successful synthetic techniques but also on effective characterization techniques to facilitate accurate analysis of these complex macromolecules. [18]

In this review, the overall framework of the MS and MS/MS analysis of synthetic polymers into its main components is evaluated. We discuss the fundamentals of polymer analysis as well as recent developments in the areas of tandem mass spectrometry, software developments, and overall future perspectives on the way to polymer sequencing. For that reason, the aim of this review is not to cover the whole polymer-MS research field, but only the parts related to sequential analysis of polymers especially focusing on MS/MS studies.

2. MASS SPECTROMETRY IN POLYMER ANALYSIS

Size-exclusion chromatography (SEC), nuclear magnetic resonance spectroscopy (NMR), liquid chromatography (LC), light scattering (LS), ultraviolet-visible spectroscopy (UV-vis), and analytical ultracentrifugation (AUC) are powerful characterization tools for synthetic polymers. In order to investigate the molar mass and the chemical heterogeneity of polymeric materials, the utilization of specific detectors was successfully performed in SEC analysis. The coupling of different types of LC separation techniques (so-called two-dimensional (2D) chromatography) was also applied in the characterization of polymers, which includes the combination of LC techniques such as SEC, liquid adsorption chromatography (LAC), liquid chromatography at critical conditions (LCCC), gradient polymer elution chromatography (GPEC), and temperature gradient interaction chromatography (TPIC) in order to gain information about different properties, like molar mass, chemical composition or functionality. [19] These classical methods (or their combinations) have served the polymer community for a long time and they will continue helping polymer scientists to analyze their polymeric substances in the future as well. Nevertheless, these traditional methods have certain limitations and drawbacks, even 2D analysis techniques are not sufficient to solve the problems in the characterization of complex polymer systems. For that reason, current research in polymer science and engineering has to move from these traditional methodologies to sophisticated analytical strategies in which MS-based techniques play a crucial role. The molecular complexity of polymers requires new technologies and approaches to provide structural insights. We believe that the analysis of synthetic polymer samples presents special challenges and limitations because of their complex structure and MS-based techniques, as powerful analytical tools for the identification of the chemical composition, structure and molar mass, can provide solutions for polymer science community.

MS techniques have gained importance in the field of polymer science and characterization, since the development of soft-ionization techniques such as electrospray ionization (ESI) [20] and matrix assisted laser desorption/ionization (MALDI). [21, 22] MALDI is the most commonly exploited and simplest MS technique for obtaining information from various synthetic polymers since it generates mostly singly charged species reducing overlaid charge states. MALDI has also a great tolerance for contaminations and salts, and make MS analysis with high sensitivity and speed accessible. ESI is a softer analysis technique compared to MALDI and is more appropriate for labile substances, in particular for polymers with fragile end-groups or supramolecular assemblies held together by means of non-covalent interactions. ESI leads to multiple charged species which can cause difficulties in the interpretation of the resulting MS spectra; on the other hand, it also enables the

detection of high molar mass substances achievable, which might not be detected in a single charge state due to mass discrimination effects. There are plenty of successful examples in the literature where both techniques assist polymer scientists to analyze their macromolecular structures. As evaluated in several reviews, MS-based techniques are extremely prosperous at investigating MMDs, monomer units, macromolecular architectures, side chain substituents, end-group functionalities, copolymer compositions and sequences, degradation products, polymerization mechanisms and kinetics. [9-11, 23]

Figure 1 represents a general scheme for the characterization of polymers *via* MS-based strategies. As can be seen from the outline, the characterization of synthetic polymers is highly dependent on the sample preparation methods and separation technologies. Sample preparation methods are highly important in particular for the characterization of polymer samples *via* MALDI. Finding the right choice of solvent, matrix and salt combination is essential for the successful MALDI analysis of polymers. Unfortunately, the incorrect choice of matrix and salt combination can lead to wrong results particularly for labile/fragile polymeric substances. The National Institute of Standards and Technology (NIST) provides a database [24] consisting of MALDI recipes taken generally from peer-review scientific publications for MALDI MS on a variety of synthetic polymers, but there are a lot more MALDI sample preparation methods reported in literature and one should always make efforts to identify the right combination for new polymer classes through examining all possible combinations. Solvent and salt used for the MS analysis are similarly important for the characterization of polymers *via* ESI technique. Moreover, separation techniques can simplify complex polymer samples prior to mass spectrometry analysis, but it is not required for all polymer substances. Direct analysis of polymer samples *via* MS and MS/MS (without separation techniques prior to MS) can generally provide useful information about their MMDs, end-groups, block lengths and composition, architectures as well as copolymer sequences. MS/MS can be employed in order to verify the peak assignments, to identify individual end-groups, to differentiate isobaric and isomeric species as well as to analyze the macromolecular architectures of polymers in detail. Comprehending the fragmentation mechanism of the investigated polymer class is necessary to gain additional structural information from the resulting fragmentation products. Moreover, special data interpretation software solutions can be optionally utilized for a faster evaluation of the complex MS/MS data from synthetic polymers. Complex polymer systems such as block, random, and graft copolymers, (hyper) branched polymers, cyclic as well as star-shaped polymers can be analyzed by using the approaches shown in Figure 1.

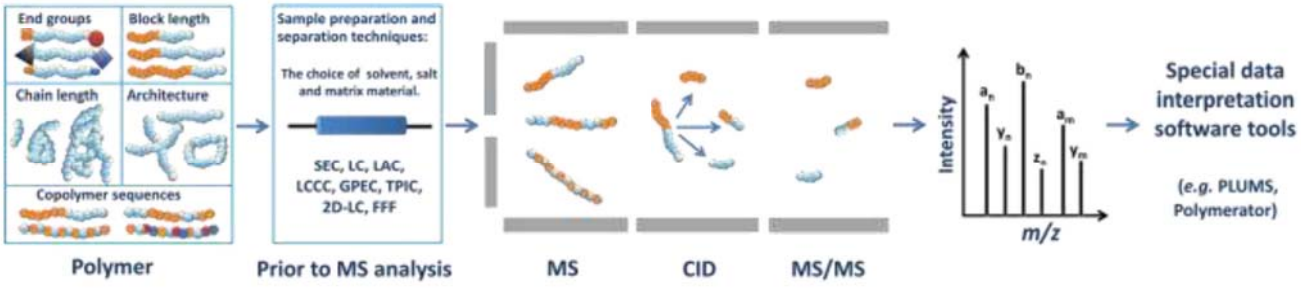

Figure 1 "Polymeromics": General outline for the characterization of polymers *via* mass spectrometry-based strategies. Polymer samples can be fractionated by various separation techniques prior to MS analysis (not obligatory). Mass to charge (m/z) ratios are measured from polymer ions that pass the collision cell without fragmentation in the mass spectrometer (MS). Specific ions are selected for collision induced dissociation (CID) experiments and the resulting fragment ions are measured in the second mass analyzer in tandem mass spectrometry (MS/MS). Special data interpretation tools can be optionally utilized for a faster evaluation of the obtained data.

As discussed above, the application of these strategies (*e.g.*, sample preparation, separation, ionization, data acquisition, and data analysis) differs depending on the sample complexity and the goals of the polymer analysis. But in most cases, tandem MS analysis provides immense information about complicated macromolecular structures. In particular together with special software tools, tandem MS can assist the structural analysis of intricate polymeric materials. In the next section, the details of tandem MS analysis will be provided along with the characteristic strengths and limitations of the polymer analysis *via* MS-based strategies. We believe that with the continuous developments in the field of tandem MS these techniques will be indispensable tools in polymer science and engineering in the future in particular for the analysis of sequence-controlled polymers.

3. TANDEM MASS SPECTROMETRY FOR STRUCTURAL CHARACTERIZATION OF POLYMERS

MS/MS is an extremely important tool for the detailed analysis of macromolecular structures and one of the main contemporary development fields in current MS research approaches. The potential benefits of MS/MS are the structural information on various polymeric materials, which is gained from the characteristic fragmentation patterns in MS/MS, complementary to that from single-dimensional MS. [25] As mentioned earlier, OMICS sciences rely on the sequential analysis of biomacromolecules by MS/MS. This method could similarly assist in the determination of sequences of synthetic copolymers. The availability of reference spectra

is critical for a meaningful analysis of the obtained copolymer fragment distributions. After a reference spectrum is determined, the chemical formula for most fragmentation product ions can be deduced applying knowledge of recognized mechanisms. In order to suggest fragmentation pathways of copolymers and to identify the nature of product ions, MS/MS spectra of the corresponding homopolymers must be investigated in detail. A characteristic fragmentation pattern can be defined for a certain type of synthetic polymer and used as a reference to characterize unidentified polymers within the same class. However, the fragmentation pathways of the same class of synthetic polymers may differ if fragile end-groups are present, for example, in case of polymers obtained from different controlled radical polymerization (CRP) techniques. Initiating and/or terminating end-groups can significantly influence chain-end and in-chain bond cleavages of synthetic polymers. As a consequence, it is extremely important to scrutinize the same polymer class with different end-groups to determine the differences in fragmentation pathways and to provide sufficient knowledge about the fragmentation characteristics of each homopolymer class in order to establish libraries of polymer fragmentation pathways. In addition, several studies showed that cationization agents influence the fragmentation pathways as well. Hence, accomplishing successful sequential copolymer analysis by MS/MS requires a good understanding of the fragmentation characteristics of the corresponding homopolymers. Therefore, polymers *i)* functionalized with different end-groups, *ii)* ionized with various cationization agents, and *iii)* analyzed under different energy conditions have to be investigated in detail. The fragmentation pathways of most common synthetic polymer ions have recently been reviewed by Wesdemiotis *et al.*. [26] In this section, our aim is to present the utilization of MS/MS analysis on synthetic polymers for different purposes such as the characterization of end-groups functionalities and architectures, copolymer compositions and comonomer sequences, as well as the application of energy-dependent MS/MS experiments to gain detailed knowledge on the way to a full sequential analysis.

3.1. Structural characterization of end-group functionalities and architectures of polymers *via* MS/MS

Advanced MS-based techniques provide extensive structural information about synthetic polymers. End group functionalities of a wide range of polymers can be straightforwardly determined by these techniques. In particular, ESI has the advantage of providing softer ionization of the large polymer molecules with complete preservation of end-group functionalities, because it generally causes less in-source fragmentation compared to the MALDI technique. The loss of the end group functionalities is often observed in MALDI leading to information loss in the case of mechanistic investigations and in the verification of the end-group fidelity.

However, these difficulties in MALDI analysis can be surmounted by the right selection of the applied conditions for sample preparation (*e.g.*, matrix, solvent, and salt) and this technique can be successfully exploited in end-group determination studies. Furthermore, MS techniques can also be utilized for the characterization of complex polymeric architectures such as cyclic polymers, star-shaped polymers, and dendrimers.

There are many successful studies especially on the end-group determination *via* MS/MS in the past decade. For example, Jackson and coworkers applied the MS/MS technique in order to analyze various homo- and copolymer classes with different end groups (*e.g.*, polyethers, poly(alkyl methacrylate)s and polystyrenes). The obtained results from their investigations elucidated combined structural/conformational information about these polymer classes. Accurate MS/MS measurements have enabled the collection of end group and first sequence information. [27-31] Weidner *et al*. applied MALDI CID MS/MS for the end group characterization of complex copolyesters and distinguished between cyclic and linear oligomers. The end groups of isomeric linear oligomers could be easily detected due to the formation of characteristic product ions. Complex copolyesters sequences were also determined by fragmentation analysis. [32] Polce *et al*. studied polystyrene (PS) oligomers with different sizes and initiating or terminating end groups *via* MS/MS analysis. PS with labile and robust end groups were compared and the influences of the nature of the end groups were investigated in detail. The authors observed that the changes in the PS backbone structure had a significant effect on the resulting fragmentation mechanisms. [33, 34] De Winter *et al*. performed experimental and theoretical investigations on CID of isomeric sodium cationized polylactides. The authors extended the ability of MS/MS in the context of end-group identification for polymer ions to the possibility to differentiate between regioisomeric end-groups. The examination of MS/MS data allowed the distinction between the three isomeric end-groups. The intermediacy of an ion/neutral complex was proposed to explain the observed fragmentation products and some theoretical calculations were performed to support the suggested structures. [35] During the past few years, Schubert and coworkers have studied several pharmaceutically important polymer classes such as poly(ethylene glycol)s (PEG), poly(2-oxazoline)s (POx), and poly(ethylene imine)s (PEI) in order to investigate the effects of different end-groups on the polymer fragmentation mechanisms *via* various MS/MS techniques utilizing ESI, MALDI and APCI. The authors have found a significant influence of the end groups on the fragmentation mechanisms of all studied polymers and they gathered significant structural information about these polymers from MS/MS investigations. In general, the scientific objective of these authors is to build tandem MS libraries to provide certain knowledge about the fragmentation mechanisms of synthetic polymers and to use this knowledge for directed applications. [18, 36-39] Song *et al*. reported a method for the end-

group analysis of methacrylic (co)polymers by LC-ESI-MS/MS. The characterization of end-groups in both methacrylic homo- and copolymers demonstrated that β-scission and radical transfer to the solvent play a crucial role in the polymerization. Isobaric polymers with structural differences (end group functionality) were simply identified by applying different isolation windows in high-resolution and high accuracy MS/MS experiments when an LC-MS experiment alone is not sufficient for such analysis. The authors stated that this method is applicable to PMMA (co)polymers and can be extended with some modifications to other homo- and copolymer systems. [40]

Recent studies demonstrated that the utilization of ion mobility (mass) spectrometry (IMS or IMMS) separation together with tandem MS can enable the fast separation and characterization of polymer compositions in complex polymeric structures to gain insight into individual end groups and isomeric architectures as well as comonomer sequences. IMMS allows the separation *via* charge state and/or architecture, enabling the identification of secondary or overlapping components indistinguishable in the total MS spectrum. IMMS offers several benefits such as a better signal to noise ratio (S/N), isomer separation, and charge state identification. This technique was first introduced by Clemmer *et al.* for the analysis of naked cytochrome c ions in the gas phase and later applied for synthetic polymers. [41-43] Current developments in IMS have shown a capacity for probing polymeric architectures and supramolecular structures. For instance, Trimpin and Clemmer have assessed the differences at the molecular level in complex polymer mixtures almost instantaneously using a prototype multidimensional IMMS instrument. The authors reported bulk activation/fragmentation strategies which provide signatures of structural characteristics allowing effortless recognition of minor differences in blends and copolymers, even as structural isomers and from a quantitative perspective. The IMMS data provided a visual pattern that is satisfactorily distinctive; as a consequence, computer-aided pattern recognition can be used to address process control and regulatory issues in the future. [42] Hilton *et al.* employed the combination of IM with MS/MS to separate and differentiate between polyether oligomers with the same nominal molar masses. PEGs with the same nominal *m/z*, but with different structures, were separated using this technique. The IM MS/MS data was used to identify the backbone and end groups of the four individual polyethers in the two sets of isobaric mixtures. The MS/MS data from the resolved oligomers enabled a detailed structural characterization of the polyether mixtures to be completed in one experiment. [44] Li *et al.* reported in their recent study that MS analysis of synthetic polymers can be improved using IMS. In this contribution, the authors showed that MS/MS combined with IMS separation provides valuable insight into the binding interactions in supramolecular assemblies and on the structures and conformations of a poly(α-peptoid) resulting after NHC elimination. The complete analysis inside the mass spectrometer allows a

fast, sensitive, and cost-effective elucidation of the polymer composition, structure, and architecture without prior derivatization, separation, or degradation. At the same time, this study emphasizes that although MS plays a fundamental role in synthetic polymer analysis, the measurement process itself might alter the analyzed substances generating misleading data; the utilization of more than one technique is recommended to recognize such changes. [45] Scionti *et al*. discussed the combination of multistage MS with LC and IMS separation for the analysis of synthetic polymer in their recent article. The effectiveness of the IM MS/MS technique was demonstrated for various polymeric substances in order to gain insights into individual end groups, isomeric architectures and comonomer sequences. [46]

A comparison study for the structural characterization of polymers synthesized *via* different controlled radical polymerization (CRP) techniques was recently reported by Altuntas and coworkers. [47] In this study, the detailed characterization of poly(methyl acrylate)s (PMA) using different MS/MS techniques (ESI, APCI, and MALDI MS/MS) was performed to provide insights into the macromolecular structure (Figure 2). The labile end-groups were determined based on the different fragmentation behavior in the CID. The fragment ions in the upper mass region which are closer to the precursor ion provided the best information about the end-group cleavages. ESI and APCI techniques were found to be better suited for the analysis of the end-group functionalities of CRP-prepared polymers; however, MALDI was found to be superior to obtain molar mass information about these polymers. Therefore, the authors suggested using all three MS techniques to gain complete information about CRP polymerizations.

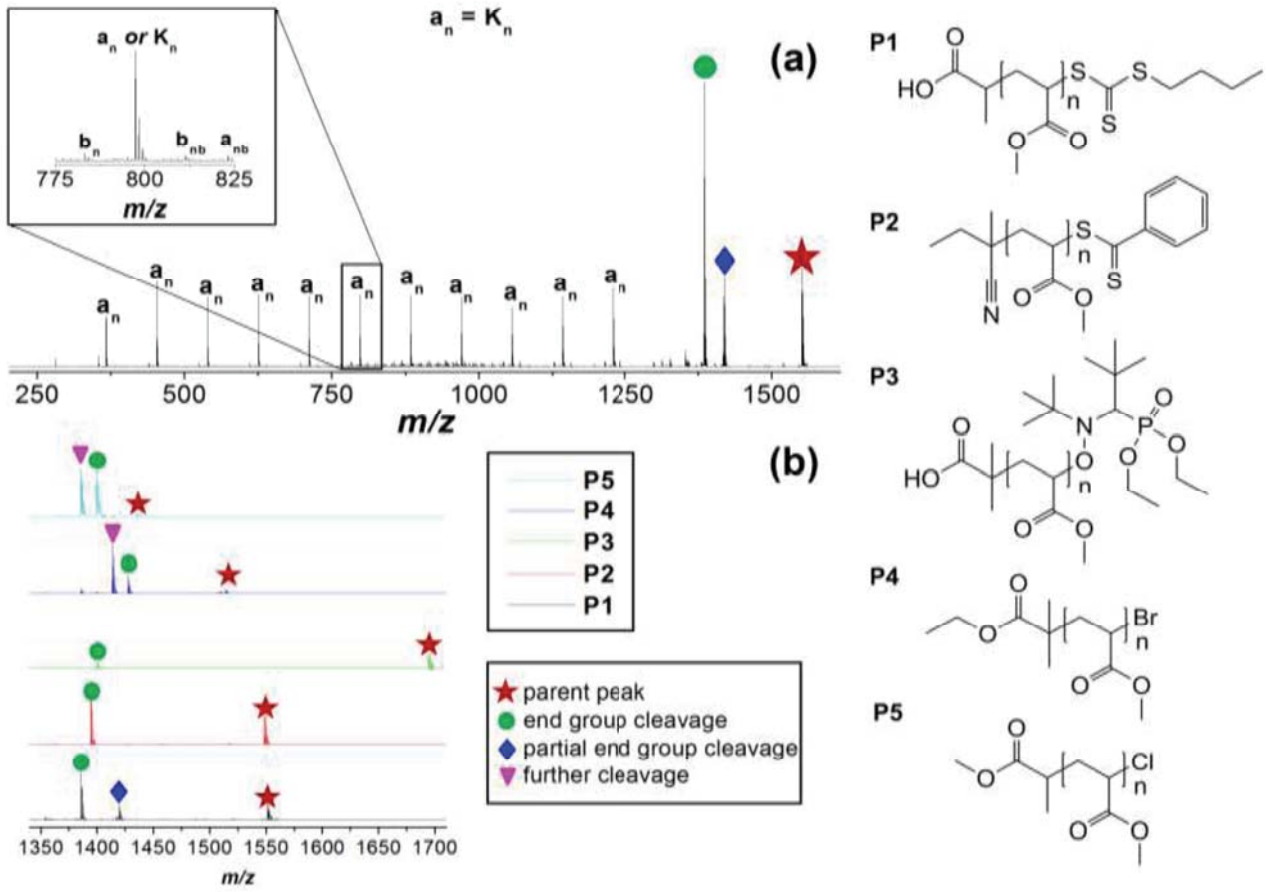

Figure 2 (a) ESI-Q-TOF tandem mass spectra of the **P1** (*m/z* 200-1700), (b) the comparison of the fragmentation behavior due to the different starting and end-groups of all investigated homopolymers **P1**-**P5** (upper mass region). Reproduced from Ref. [47] with permission from Wiley Periodicals, Inc..

Towards architectural characterization, MS techniques have proven to be vital characterization tools by many successful studies. For example, detailed characterization studies of linear and branched polyacrylates with various side groups were performed using MS/MS technique by Chaicharoen *et al*.. It was observed that the fragmentation patterns depend on the size of the ester alkyl pendant group. Polyacrylates with small alkyl groups decompose *via* free radical chemistry, initiated by random homolytic C-C bond cleavages along the polymer chain. The formed radical ions dissociate further by backbiting rearrangements and β scissions to generate a distribution of terminal fragments with one of the original end groups and internal fragments with several repeating units. Polyacrylates with small alkyl groups dissociate generally by charge-remote 1,5-H rearrangements that convert COOR to COOH groups. Moreover, the determination of the branching architecture was possible due to the fact that the unique alkenes and alcohols are cleaved from ester groups at

the branching points and the rearrangements yield fragments characteristic of the branch sizes. [48] In another study, differentiation of linear and cyclic polymeric architectures by MALDI MS/MS was achieved by Yol and coworkers. Different architectures resulted in significantly different fragmentation products *via* MS/MS experiments (Figure 3). For linear polymeric architectures, the separated radicals depolymerize extensively by monomer losses and backbiting rearrangements, leading to low-mass radical ions and much less abundant medium- and high-mass closed-shell fragments that contain one of the original end groups, along with internal fragments. On the other hand, for cyclic polymeric architectures, depolymerization is less efficient, as it can readily be terminated by intramolecular H-atom transfer between the still interconnected radical sites. Simple examination of the relative intensities of low- *vs.* high-mass fragments allows a conclusive determination of the macromolecular architecture, while full spectral interpretation reveals the individual end groups of linear polymers or the identity of the linker used to form the cyclic polymer. [49]

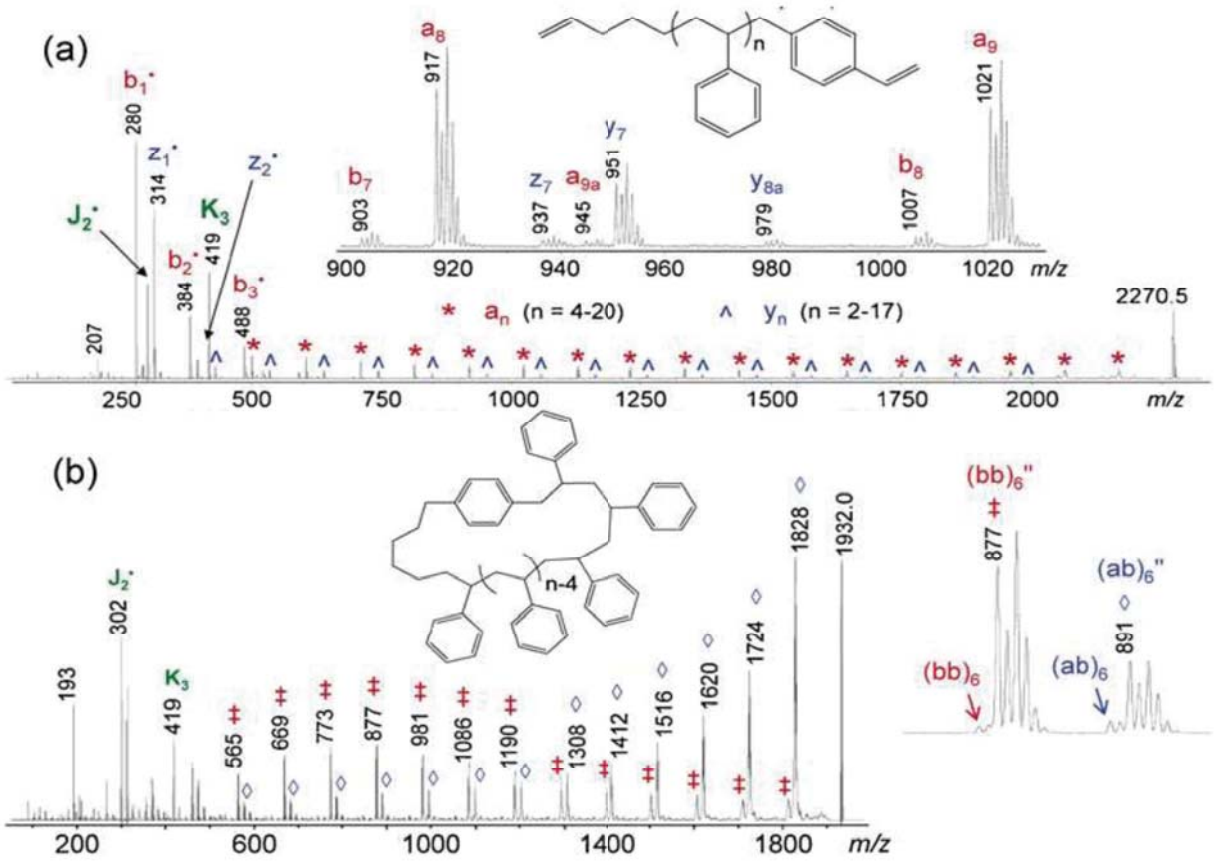

Figure 3 MALDI MS/MS spectra of (a) the silverated 19-mer of linear α-4-pentenyl-ω-(p-vinylbenzyl) polystyrene (*m/z* 2270.5); (b) the silverated 23-mer from the macrocyclic PS obtained by metathesis ring-closure of α-4-pentenyl-ω-(p-vinylbenzyl) polystyrene (*m/z* 2658.5). Reproduced from Ref. [49] with permission of the American Society for Mass Spectrometry.

All of the reviewed studies in this section confirmed the significance of MS/MS methods for the end group functionality and architecture analysis of synthetic polymers on the way to sequential analysis of these materials. In particular, the combination of MS/MS with LC or IMS offers many advantages. The knowledge gained from all of these studies can be collected in polymer MS databases and web interfaces like in other OMICS sciences to share, compare and verify the obtained polymer MS data towards a complete polymer sequencing. This idea will be further discussed comprehensively in the last chapter.

3.2. Elucidation of copolymer compositions

The final physical and chemical properties of copolymer materials are determined by the composition and sequence distribution of the monomer units across the copolymer chains. Therefore, it is important to determine the chain length distribution and average composition of the polymers as well as to elucidate the sequence distribution for a good understanding of the correlation between structure and properties. MS/MS can be utilized to separate and dissociate oligomer compositions in copolymer mixtures to obtain sequence specific fragments. The extensive structural details can be obtained by MS/MS investigations of complex (co)polymer mixtures, and it provides a powerful method for relating these synthetic variables to the physical properties that are crucial for specific applications. For this purpose, MS/MS (or MS^n) experiments are the key methods for elucidating detailed structural and sequence information of copolymers. The obtained results are required to depict chain ends of unknown substances and to distinguish block copolymers from random copolymers. In view of the fact that each co-monomer reveals a different dissociation behavior, fragmentation pathways along the chain generally permit copolymers to be sequenced.

In the last decade, a number of studies were published on the characterization of copolymers aiming at the sequential analysis by MS and MS/MS. Montaudo published a review article that focused on copolymer composition and average molar mass determination as well as on the benefits of coupling MS with separation techniques. The author demonstrated the great potential of MS to determine all copolymer quantities by simply recording the copolymer's mass spectrum and interpreting the information contained in it. A summary of the studies on the mass spectra of copolymers were provided along with the special methods on copolymer MS data analysis. The advantages and limitations of the copolymer characterization *via* MS were discussed in detail in this review article over a decade ago. [50] However, MS/MS studies were not recognized for

compositional and sequential analysis of copolymers in this work. Hence, our aim here is to discuss successful examples of MS/MS studies on copolymers and to illustrate the usefulness of this technique for the sequential characterization of copolymers.

Cerda and co-workers have separated a poly(ethylene/propylene glycol) (PEG/PPG) copolymer mixture using Fourier transform MS and dissociated the copolymer into sequence-specific fragment ions with electron capture dissociation (ECD). Contrary to the indicated triblock "PEG/PPG/PEG" sample description of this commercial surfactant, all observed oligomers consisted mainly of diblock PEG/PPG structures, so that their termini differ significantly in hydrophobicity, as expected for a surfactant. Although lacking reference copolymers of well-known isomeric structure, the extensive structural details were obtained by ECD MS/MS from such complex copolymer mixtures proving the usefulness of this technique for connecting synthetic variables to their properties. [51] Przybilla *et al.* exploited post-source decay matrix-assisted laser desorption/ionization time-of-flight (PSD-MALDI-TOF) to determine the structural information from a poly(ethylene oxide)-*b*-poly(p-phenylene ethynylene) diblock copolymer (PEO-*b*-PPE). MS/MS spectra revealed a main cleavage of the copolymer chain between the two blocks (cleavage of the ester functionality situated between the two blocks). As a result, the length of each block could be explicitly determined. [52] Arnould *et al.* characterized a polyester copolymer produced by step-growth polymerization using MALDI MS and MS/MS and their study generated detailed insight about the composition, end groups, molar mass, and sequence of the product. MS/MS experiments on low-mass oligomers indicated that sequence information can be gathered from the observed fragmentation patterns and it was found that specific sequences are favored in step-growth copolymerizations. [53] Jackson *et al.* studied ethylene oxide/propylene oxide (EO/PO) copolymers *via* ESI MS/MS. The detailed assessment of fragmentation pathways has enabled end group and sequence information to be obtained. The combination of IMS with MS/MS has also been applied to facilitate the generation of structural information in case of EO/PO copolymers. [27] In a study from Rizzarelli *et al.*, poly(ester amide)s were characterized by PSD-MALDI-TOF MS and MS/MS. The authors provided information on the ion fragmentation mechanisms of the poly(ester amide) chains. In addition to the ions observed in the PSD-MALDI mass spectra, several new abundant product ions in the low mass range are present in the MALDI-TOF/TOF MS/MS spectra. These new ions were diagnostic fragments and enabled the presence of random sequences of ester and amide bonds in the poly(ester amide)s samples. [54] Terrier *et al.* have investigated EO/PO block copolymers by MS/MS and obtained valuable information regarding the copolymer sequences. CID of ammonium adducts of various linear triblock (*i.e.*, PEO-*b*-PPO-*b*-PEO and PPO-*b*-PEO-*b*-PPO) and glycerol derivative diblock copolyethers (*i.e.*, gPEO-*b*-PPO and gPPO-*b*-PEO) produced by ESI was studied under low-

energy conditions. Copolyethers with the same composition in each repeating unit but with inversed block sequences (*i.e.*, PEO-*b*-PPO-*b*-PEO *vs.* PPO-*b*-PEO-*b*-PPO and gPEO-*b*-PPO *vs.* gPPO-*b*-PEO) were distinguished with characteristic fragment ions. CID spectra provided accurate information about the block lengths in the case of linear copolymers. MS/MS experiments permitted the explicit distinction of linear or glycerol derivative copolyethers with the same composition in repeating units but with different block sequences. [55] Mass *et al.* has published a study about the analysis of methylenebiphenyl diisocyanate/toluene diisocyanate (TDI–MDI) oligomers obtained by hydrolysis of a crosslinked polyurethane (PU) regarding the oligomer distribution by MALDI-TOF-MS and the monomer sequence in the different oligomers by CID experiments. Specific product mass peaks were identified (that were characteristic for random and blocky monomer sequences) by selecting suitable oligomer precursor ions with different monomer sequences out of the total oligomer distribution. As a result, a MS method has been presented for the important class of PUs that is selective towards the number and type of monomer units as well as towards their sequence in the oligomer chain. [56] Wienhöfer *et al.* analyzed the sequence and composition of the polymeric structures (synthesized *via* the copolymerization of methyl methacrylate (MMA) with a few mol % styrene) primarily at the terminal ends by applying MS-MS experiments. In this study, the utilization of the MS/MS technique also allowed the successful characterization of various co-oligomers sequences. [57]

The combination of high-resolution Fourier transform mass spectrometry (FTMS) and MS/MS provides a powerful analytical tool for the characterization of complex polymer systems, in particular for copolymers. Simonsick and coworkers reported a good method utilizing FTMS together with MS/MS for the detailed structural characterization and sequencing of polymers. The method was used to separate and identify two by-products of the polymerization of 12-hydroxystearic acid (HSA) and stearic acid (SA) to provide precise information about the exact location of caprolactones on the tris(2-hydroxyethyl)isocyanurate (THEIC) molecule, and to sequence a glycidyl methacrylate/methyl methacrylate (GMA/MMA) copolymer. [58, 59] Koster *et al*. studied synthetic homo- and copolyesters *via* ESI Fourier transform ion cyclotron resonance (FTICR) MS and MS/MS. Several copolymers were investigated in order to gain sequence information of the copolyester oligomers. Sequence-specific fragments of two out of the three copolyester sequences that theoretically can exist were experimentally scrutinized. [60] Miladinovic *et al.* has also applied the usage of a tandem FTMS instrument to scrutinize block and random copolymers in a sequential manner. Their research enabled rapid differentiation of block and random copolymers by a suitable analysis of their fragmentation patterns. The authors compared the utilization of quadrupole collision-induced dissociation (QCID)-FTMS and electron capture dissociation (ECD)-FTMS to fragment polyacrylate and polyether oligomer ions formed by ESI

or MALDI. Identification of fragment ions with monomer losses delivered insights into each copolymers nature; as a consequence, random and block copolymers can be differentiated by QCID-FTMS technique. The ECD-FTMS spectra of the polyacrylates showed only losses of single methyl and butyl side-groups for copolymers as well as single methyl groups for homopolymers; therefore this specific behavior of polyacrylates during the ECD process could be useful for the side-group determination in unknown copolymers. [61]

Schubert and co-workers characterized several poly(2-oxazoline) (POx) block copolymers by MALDI-TOF MS/MS and ESI-Q-TOF MS/MS. [62] This study confirmed the block copolymer structure and elucidated successfully the fragmentation behavior within the polymer chain. In particular, ESI-Q-TOF MS/MS data have provided important structural information on the copolymer sequence due to the depolymerization mechanism of the ESI-generated copolymer ions. The depolymerization mechanism (unzipping or monomer evaporation) of POx block copolymers could be easily monitored for the identification of copolymer sequences (Figure 4). The protonated precursor ion of the block copolymer had a tendency to lose initially its terminating (ω) end-group, and later one monomeric unit at a time starting from the one attached to the ω end-group. The fragments exhibited subsequent losses of one monomeric unit of EPOx (169.14 Δ*m/z*) until it was diminished in the block. After that, the depolymerization mechanism of the EtOx (99.07 Δ*m/z*) monomer units could be followed through the MS spectra. A complete depolymerization of the each block allowed the determination of both its end-group and the segment sizes of the blocks. [62]

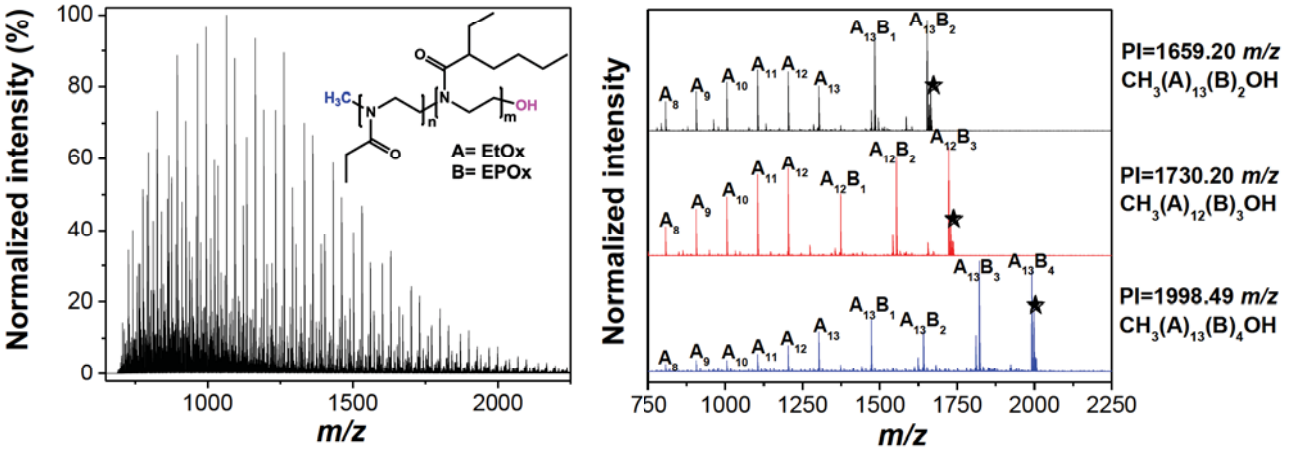

Figure 4 ESI-Q-TOF MS and MS/MS spectra of p(EtOx-*b*-EPOx) copolymer. Reproduced from Ref. [62] with permission from Wiley Periodicals, Inc..

Within the last few years, Charles and coworkers have contributed to this field with several studies focusing on the detailed characterization of various copolymer systems using MS/MS experiments such as poly(ethylene oxide)/polystyrene block copolymer (PEO-*b*-PS) block copolymers [63-66], poly(methacrylic acid)-poly(methyl methacrylate) copolymers [67-69] and poly(methylhydrosiloxane)-*co*-(dimethylsiloxane) copolymers [70]. The authors have achieved very useful complementary structural information by performing MS/MS experiments on these different copolymer structures. In most cases, the detection of the various product ion series with possible co-monomeric compositions confirmed the random and/or block nature of the various investigated copolymer samples.

All aspects considered, the findings from all of these successful studies are highly encouraging that the sequence determination of other complex copolymers will be possible by the utilization of MS/MS techniques in the future. Certainly, these expectations depend not only on the instrumental enhancements but also on software developments in the near future. At the moment, MS/MS experiments can be generally applied to lower mass oligomers from 3,000 to 5,000 *m/z* due to the current instrumental limitations. In addition, there is no available software tool to interpret complex MS/MS data of copolymers in an automated manner like in OMICS fields. Hopefully, with new developments in this field, MS/MS experiments can be applied to many complex polymeric systems to elucidate structure-activity relationships.

3.3. Energy-dependent collision induced dissociation (CID) and characteristic collision energy (CCE or CE_{50}) in tandem MS analysis of polymers

MS/MS is a powerful method for the structural characterization of synthetic polymers. [25, 26] This method can significantly facilitate the existing analytical methods (*e.g.* separation systems) that face the challenging complexity of synthetic polymers. The experiments can be performed upon precursor ions of interest in order to obtain additional structural information from the fragmentation patterns and to understand the fragmentation pathways of various polymer classes. Nevertheless, a standard procedure to deal with polymer sample complexity is required that allows to distinguish derivations of structural information of synthetic polymers from the observed fragmentation patterns. In order to achieve this goal, a good interpretation of the fragmentation behavior of synthetic polymers in different excitation conditions and as a function of their composition is needed. Therefore, it is necessary to analyze polymers *via* energy-dependent CID experiments to explore the dependence of the fragmentation patterns as a function of the collision energy. [71]

MS/MS can be utilized for the examination of the amount of energy required to drive the reaction fast enough to observe fragmentation products. Characteristic collision energy (CCE, CCV, CE_{50}, or E_{50}, characteristic collision energy is the collision energy value to achieve 50% fragmentation) values for synthetic polymers can be determined by exploiting energy-dependent MS/MS experiments. Within the last years, several polymer classes were investigated in detail by utilizing the CCE values such as polyethers, polymethacrylates, polyesters, polysaccharides, and poly(oxazoline)s. [72-79] In this kind of studies, attaining survival yield (SY) curves from the energy-dependent CID data provides a convenient way of presenting all of the information on the CID processes undergone by a specific type of polymer ion. The SY method is generally employed in the MS field as a tool for assessing the precursor ion stability and the internal energy. CCE values can be obtained from the SY curves in a straightforward manner and these values can be exploited to discriminate between different compounds (even structural and positional isomers). The SY method can also provide insights about the energy requirements of the fragmentation pathways of synthetic polymers. Therefore, it shows potential as a valuable method for the identification and the discrimination of polymers with different functionalities or backbones. For example, Nasioudis *et al*. used the CCE values to discriminate various polymer classes such as polyethers, poly(methacrylate)s, polyesters, and polysaccharides. Good linear correlations between the CCE values and precursor ion masses (PIM) were found for all investigated polymers in this study (Figure 5). This technique has also allowed to study the effect of end groups on the CCE values. The differences in the CCE *vs*. PIM lines seem to be associated to differences in the type of intermonomer bond, the difference between linear and cyclic structure of the polymer and the end groups of the polymer. [75] Eventually, the obtained linear correlation allows the utilization of the CCE *vs*. PIM lines for the development of standardized fragmentation methods of polymers.

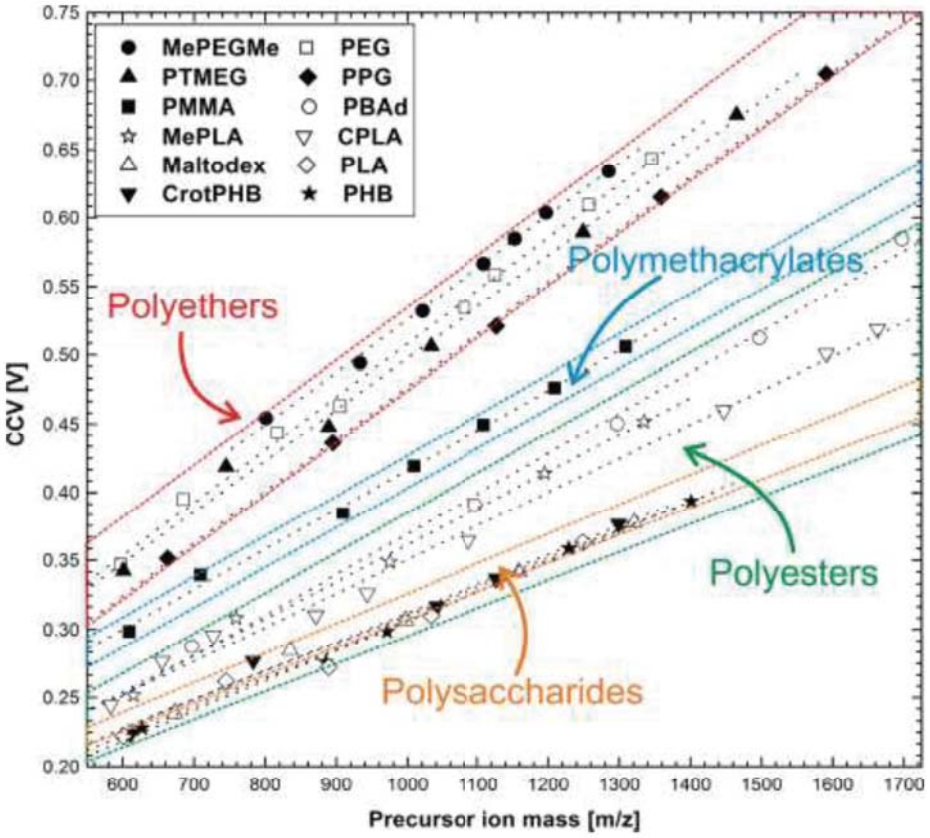

Figure 5 Characteristic collision voltage (CCV or CCE) *vs.* the precursor ion masses of the studied singly lithium cationized polymers. The data points per polymer were linear fitted and grouped into four groups: polyethers (red), polymethacrylates (blue), polyesters (green), and polysaccharides (orange). Reproduced from Ref. [75] with permission of American Chemical Society.

More importantly, this method could also be applied for the characterization of polymer/biomolecule complexes in order to examine the stabilities of these substances in the gas-phase. Smiljanic *et al*. investigated complexes between low molar mass poly(ethylene imine) (PEI) and single-stranded oligodeoxynucleotides (ODNs). The CCE values of the five PEI–ODN complexes studied indicate the intrinsic (gas-phase) stabilities are similar to the solution stabilities of these polyplexes. The obtained results suggested that the evaluation of the dissociation energetics of PEI-ODN complexes *via* energy dependent MS/MS experiments provides insights that can be valuable for the planned *in vivo* application of the polymers. The information gained from CCE values can help to understand the delivery profiles of different ODNs. The particularly high binding affinity of d(TTTTT) (a type of pentadeoxynucleotide) was observed compared to the other ODNs (Figure 6). The findings suggested that sequence-specific delivery systems could be developed by an appropriate design of the size and composition of the polymeric delivery vehicle. [80] The author stated that their future studies will focus on

examining larger polyplex constituents, other cationic polymers, ODNs with different sequences (but rich in specific nucleobases) and single- vs. double stranded ODNs. Ultimately, scrutinizing the effect of such determinants on the polyplex composition and stability may lead to the design of more efficient transfection vectors.

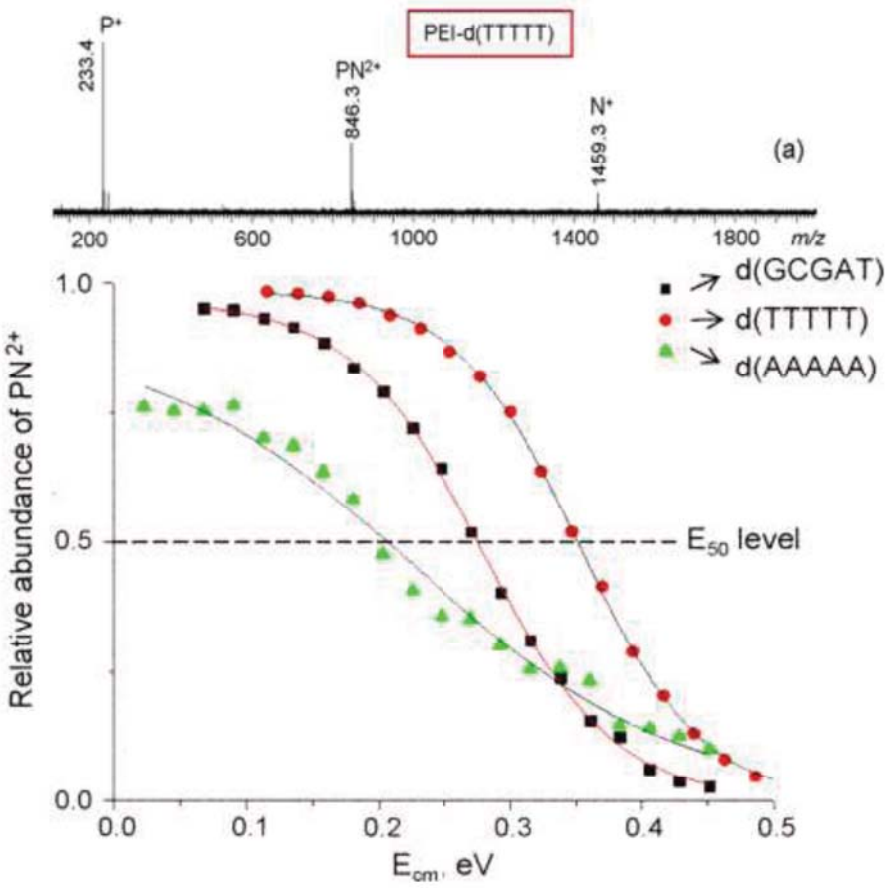

Figure 6 Fragmentation efficiency curves (relative abundance of $PN2^+$ precursor ion *vs.* center-of-mass collision energy) of polyplexes containing a PEI with 5 repeating units and d(TTTTT), d(AAAAA) or d(GCGAT). Reproduced from Ref. [80] with permission of Elsevier.

Hopefully, this method will be applicable to differentiate more complex polymer architectures such as graft, block or random copolymers and other polymeric structures on the way to a sequential analysis. For that reason, MS/MS experiments should be performed in the future covering a wide range of collision energies to build a complete library of MS/MS reference spectra for synthetic polymers. The importance of the fragmentation ion libraries for synthetic polymers will be discussed in the next section in detail.

4. SOFTWARE STUDIES ON THE WAY TO A SEQUENTIAL ANALYSIS OF POLYMERS

As previously mentioned, the analysis of synthetic polymer samples presents special challenges and limitations because of their complex polydisperse structures. MS-based techniques (MS and MS/MS) combined with proper separation techniques can provide solutions for the polymer science community. The utilization of MS/MS to analyze synthetic polymers is particularly attractive, because other techniques cannot provide detailed structural information by allowing comprehensive sequence information to be derived from the mass spectra. Currently, possessing expert knowledge and certain skills are required in the manual interpretation of the MS/MS data obtained from synthetic polymers. On the other hand, the utilization of MS-based strategies in polymer science has recently become routine. As a result, the number of novice user of these strategies has increased in the polymer science community. The major difficulty at this point is the transformation of tandem mass spectral data of synthetic polymers into chemically valuable information with a minimum involvement of the user, preferably through automated interpretation of the complex (co)polymer fragmentation patterns. The advances in software development and the large availability of open source software solutions for complicated MS/MS data in OMICS sciences have greatly assisted many scientists in various research fields, especially in proteomics. [2, 5, 81, 82] In a similar manner, polymer researchers can also benefit from the development of such software tools for the analysis of polymers *via* MS/MS. Therefore, in order to achieve insights into the macromolecular structure with MS-based methods, it is important to develop special data interpretation software tools to identify fragmentation products obtained by MS/MS as in the fields of various OMICS sciences. Even though the extreme importance of computational analysis of MS/MS data from polymers, it is unfortunately a rather embryonic field of research; this represents clearly one of the major problems in MS/MS analysis of polymers.

Up to now there are only two software tools available for the evaluation of MS/MS data from synthetic polymers. The first one is the Polymerator software which was developed by Thalassinos *et al.*. [83] This novel software aims to assist the interpretation of MS/MS data from synthetic polymers. The software is mainly focused to support the end-group determination of synthetic polymers by significantly accelerating the interpretation process of complicated MS/MS data. It provides information on the initiator and/or chain transfer agents, used to generate the polymer, and the mechanism of termination to be inferred from the data much more rapidly. The software supports polymer scientists to validate the proposed structures by automatically interpreting the data file by previously suggested fragmentation rules for synthetic polymers. The

utilization of the software provides a fast annotation of the MS/MS data, which significantly reduces the time taken for interpretation compared to manual annotation. However, this software requires the knowledge of the fragmentations pathways of the investigated polymers and information about the kind and number of main chain repeating unit, as well as the initiating group (α), the terminating group (ω) and the cationization agent. A screenshot from the Polymerator software of an annotated ESI-MS/MS spectrum from the lithiated decamer of PEG is displayed in Figure 7. The authors have later used this software tool for further studies to investigate several polymer classes such as polymethacrylates, polyethers, and polystyrenes. [30, 31, 83, 84]

Figure 7 Screenshot of software with an annotated ESI-MS/MS spectrum from the lithiated decamer of PEG. Details of annotated fragment ions are displayed by the software in the table below the spectrum. Predicted fragment ions are also detailed above (left) of the spectrum. Reproduced from Ref. [83] with permission of the American Society for Mass Spectrometry.

The second one is the PLUMS (Polymer labeling using mass spectrometry) software which was created by the group of Boecker. [85] This new software tool has been developed with certain advancement to accelerate the interpretation of MS/MS spectra obtained without requiring any further knowledge about the polymer class or the fragmentation behavior. This software simply requires an alphabetical list of elements and a peak list of the measured substance as a XML file for the assessment of the selected mass spectrum. The software determines the monomer repeating unit and the formulae for the corresponding end groups of the series of fragmentation products. Moreover, the software is independent of the operating system. The authors have exploited this software to analyze MS/MS data from various homopolymer classes such as poly(2-oxazoline)s, poly(ethylene glycol)s and poly(styrene)s. An application of the PLUMS software to interpret the MS/MS spectrum of poly(2-(1-ethylpentyl)-2-oxazoline) (pEPOx) is displayed in Figure 8. Additionally, it is possible to use this software tool for the MS/MS data obtained *via* different ionization techniques such as MALDI, ESI, and APCI. [47, 86] Boecker and Schubert are currently working on a new software tool focused on the expansion of this software including the interpretation of MS/MS spectra from block and random copolymers with a sequence analysis (*de novo* sequencing for polymers).

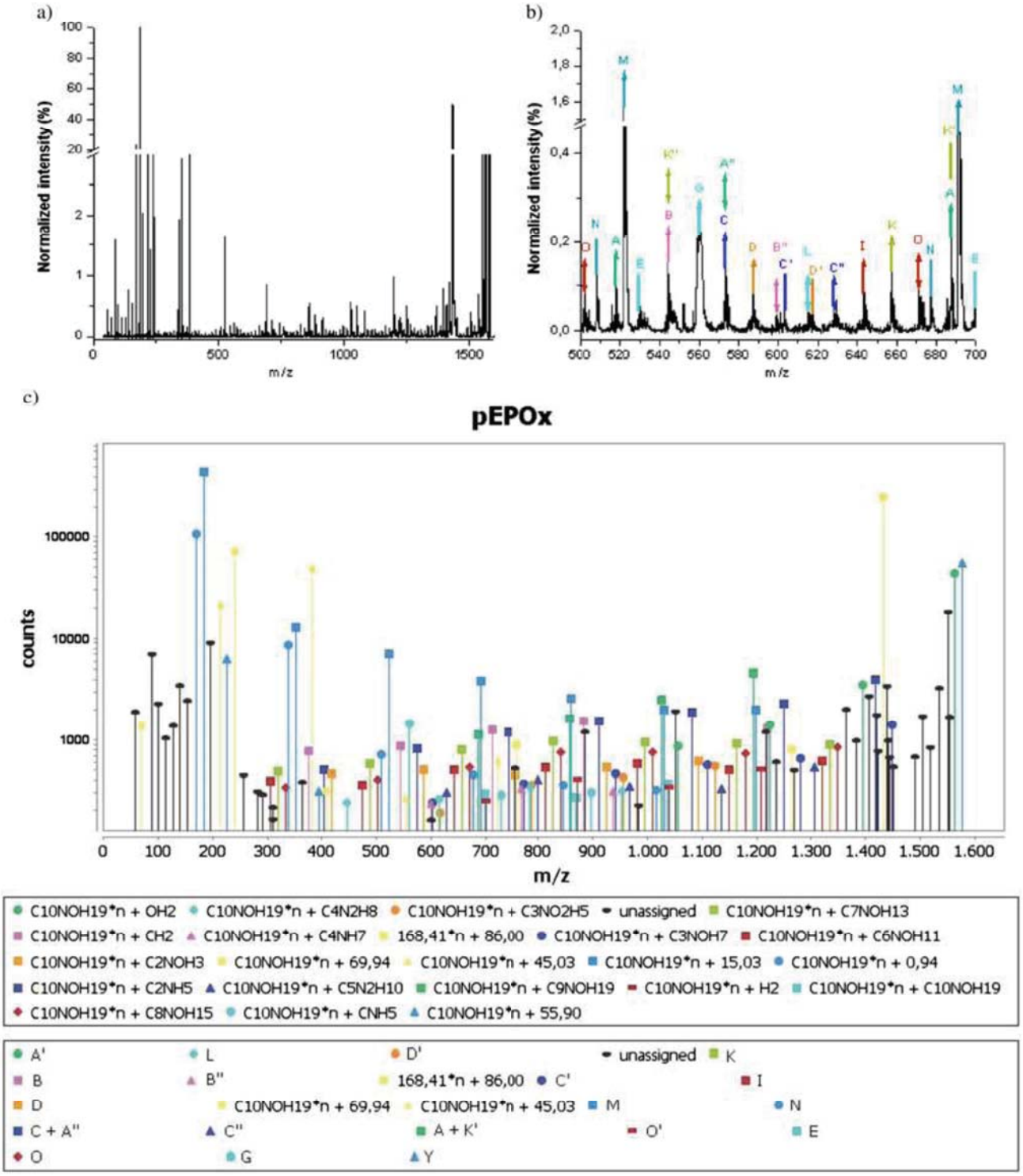

Figure 8 (a) MALDI-TOF MS/MS spectrum of the poly(2-(1-ethylpentyl)-2-oxazoline) (pEPOx) (selected precursor ion at *m/z* 1577; matrix: DCTB in $CHCl_3$, ionization salt: NaI in acetone, solvent: $CHCl_3$). (b) Zoom into the MALDI-TOF MS/MS spectrum (*m/z* 500 to 700) of the selected precursor ion of pEPOx at *m/z* 1577. (c) Results of the analysis by the PLUMS software for pEPOx. Reproduced from Ref. [85] with permission of John Wiley & Sons, Ltd..

The evaluation of the tandem mass spectra with dedicated special software tools was discussed above to show that such approaches are extremely helpful for polymer scientists for an automated analysis of complex tandem mass data. These preliminary results are indeed fascinating and open some interesting perspectives for the future developments of software tools. As expected, the future challenge will be the developments of new software tools to analyze MS/MS data from more complex systems such as various polymers with different architectures (*i.e.* branched and cyclic polymers), copolymers, and star-shaped polymers. On the other hand, the developments of new software tools alone are not sufficient to achieve the aim of polymer sequencing. It is necessary to build reference libraries for fragmentation product ions of polymers and to provide open-source MS and MS/MS data accessible for all polymer scientists. Researchers are creating a vast amount of scientific data every year and the data supply is growing very fast; however, most of the acquired data are unstructured, disconnected and constantly changing due to the developments in the studied research fields (*e.g.*, instrumental advances). The ability to access the experimental data, store it, organize it, evaluate it, and unlock its value will determine winners and losers in every field of research. The combination of scientific data and potent software tools can change data analysis from an exercise in hindsight to an analytical real-time science. A number of open-access as well as commercial tools have been developed for storage, analysis, and interpretation of proteomics, metabolomics and transcriptomics data. Similar open access tools have to be adopted in polymer science as well.

In order to obtain useful data, a variety of mass spectrometers are employed in MS-based OMICS research. Every instrument has a special design, data system and performance specifications, resulting in strengths and weaknesses for different types of experiments. However, the native binary data formats produced by each type of mass spectrometer are also different and generally proprietary. The diverse character of the data structure causes difficulties in the integration of new instruments into established infrastructure, inhibits the analysis, exchange, evaluation and publication of findings from different experiments and laboratories, and prevents the researchers from accessing data sets essential for the development of software tools. Deutsch and co-workers introduced the 'mzXML' format, an open, generic XML (extensible markup language) output of MS data and also developed an accompanying suite of supporting programs. The expectation from this format is to facilitate data management, interpretation and distribution in proteomics research. [87-91] The authors have developed this format based on XML as a common, open representation for mass spectrometric (MS), tandem mass spectrometric (MS/MS) or multiple mass spectrometric (MS^n) data. As a result of the utilization of instrument-specific converters, the mzXML format provides a universal data interface between mass spectrometers and common data analysis pipelines. We assume that the mzXML format can be similarly utilized in polymer

research *via* MS-based techniques (MS, MS/MS or MS^n) in order to form a common (open source) data analysis framework for the polymer community (Figure 9). This framework will embrace the need for a publicly accessible database of polymers analyzed *via* MS-based studies and software tools that provides the polymer community a platform to select and validate mass spectrometric strategies. The realistic scientific objectives have to be set in the near future in order to reach this goal. Ultimately, bioinformatics researchers will be released from the necessity to gain knowledge about different company specific data formats. Additionally, standard data files will permit public storage and retrieval of data over long periods of times. Eventually, mass spectral libraries for synthetic polymers will be more rapidly established in order to identify and verify polymer MS data.

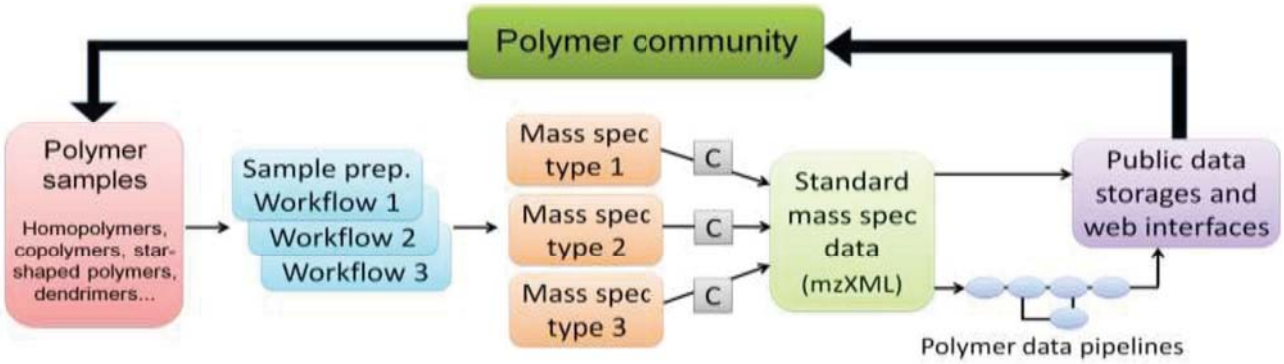

Figure 9 Open source data analysis framework for "Polymeromics". The mzXML file acts as a mediator, allowing multiple input formats to be subjected to a common data analysis pipeline. New types of instruments can be integrated into a preexisting analysis framework with only a utility (here represented by C) to convert MS native output to the mzXML format. The open structure of mzXML instance documents makes them suitable for data exchange such that, for example, they may be submitted to a data repository to support the results presented in a publication or a report. Modified from Ref. [87] and also from Ref. [92].

Taking everything into account, creating tandem mass spectral libraries for synthetic polymers is the most important task on the way to "Polymeromics". Unfortunately it is not clear at the moment when these libraries will be sufficiently large to be used as identification tools. The sensitivity and resolution of mass spectrometers will certainly continue to increase, and the analyzable content of complex polymeric systems will be expanded in the future. Both library growth and specialized software tools will be required to deal with the increase in complexity of these samples. It is unquestionable that these libraries will be very effective for the sequential analysis of polymers in the future, and the "Polymeromics" field will be one of the last Holy Grail in modern polymer science. We assume that several years of research are required to attain the ultimate goal of polymer sequencing with MS-based techniques and special software tools.

5. CONCLUSIONS AND OUTLOOK

MS-based high-throughput approaches are crucial in the comprehensive analysis of synthetic polymers. The utilization of MS-based techniques not only opens up new possibilities for overcoming the problems encountered in polymer characterization but also creates new opportunities for the use of these techniques as routine analysis methods in macromolecular engineering to monitor the synthesis of novel polymeric materials. Implementation of the MS-based strategies (*e.g.*, sample preparation, separation, ionization, data acquisition, and data analysis) differs depending on the sample complexity of polymers and the goals of the analysis. After the development of soft ionization techniques, MS-based strategies have been successfully utilized at investigating MMDs, monomer units, macromolecular architectures, side chain substituents, end-group functionalities, copolymer compositions and sequences, degradation products, polymerization mechanisms and kinetics. Software interpretation tools have also proven to be useful to evaluate complex polymer MS and MS/MS data. However, current software developments in this field are still in their embryonic stages. In order to accelerate the analysis of polymers *via* MS-based techniques, supplementary software solutions are required to handle the MS and MS/MS data in an automated manner like in many OMICS sciences (*e.g.* in proteomics). Moreover, there is a need for publicly accessible databases of polymers identified *via* MS/MS studies as well as software tools that provide the polymer community a platform to select and validate mass spectrometry targets.

Imitating Nature's unique sequential production control of large macromolecules enables active control of future's material design. Controlling the sequence of building blocks in polymers opens new ways for customizing the properties of synthetic polymers for potential applications in different fields such as in chemistry, physics, material science as well as biological and medical sciences. As a consequence, there is a large demand from polymer scientists to find proper characterization methods for a sequential analysis of synthetic polymers. Such a sequence analysis of polymers could be the next breakthrough in polymer science. In this review article, examples of published studies are used to highlight the significant impact of MS for the characterization of polymers and to illustrate the potential application of polymer sequencing in the direction of "Polymeromics". All of the reviewed MS/MS studies show that the successful sequential analysis of polymers is possible with these strategies, however there are still a large number of issues to be resolved such as the development of special software tools, the construction of reference libraries for polymer product ions, and the availability of open-source MS and MS/MS data for the polymer community. Therefore, we believe that future research approaches in this field will focus on the developments to build polymer product ion libraries

and to develop software tools to automate and simplify the creation and exploitation of data sets obtained *via* MS/MS analysis of synthetic polymers.

Acknowledgements

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Publication P2:

Electrospray ionization-mass spectrometric (ESI-MS & MS/MS) analysis of poly(2-oxazoline)s with different side groups

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Esra Altuntaş, Kristian Kempe, Anna Crecelius, Richard Hoogenboom, and Ulrich S. Schubert

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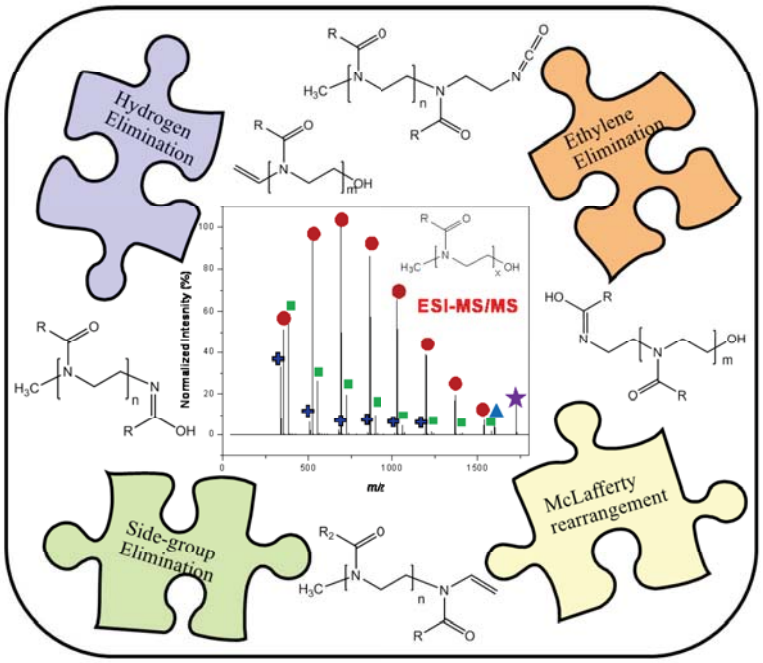
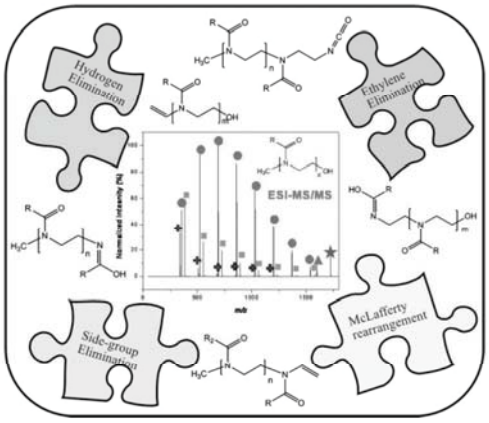


ESI-MS & MS/MS Analysis of Poly(2-oxazoline)s with Different Side Groups

Esra Altuntaş, Kristian Kempe, Anna Crecelius, Richard Hoogenboom, Ulrich S. Schubert*

ESI-Q-TOF MS has been used for the detailed characterization of various poly(2-oxazoline)s with different side groups (i.e. methyl, ethyl, 1-ethylpentyl, phenyl, *o*-difluorophenyl and *p*-*tert*-butylphenyl) to evaluate this method as structural characterization tool for the detailed analysis of poly(2-oxazoline)s. The results obtained provided an understanding of the fragmentation of poly(2-oxazoline)s, revealing the elimination of small molecules such as ethene and hydrogen in their fragmentation patterns, which are partially dependent on the side groups. Also, a McLafferty rearrangement can be a possible fragmentation route for these polymers. In detail, side group elimination was only observed for poly(2-alkyl-2-oxazoline)s, but not for poly(2-aryl-2-oxazoline)s.



Introduction

Mass spectrometry has become an important tool for the characterization of different macromolecules in recent years based on the development of electrospray ionization mass spectrometry (ESI-MS)[1] and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).[2,3] Although involving different processes in ion formation, both techniques generally allow the ionization of various macromolecules with little or no fragmentation, enabling accurate molar mass determination by making the unfragmented structure amenable to mass separation. A combination of these techniques to high-resolution mass analyzers such as quadrupole-time-of-flight (Q-TOF) instruments provides exact molar masses for the polymers analyzed with mass accuracies in the ppm region. As a result, valuable information on the chemical constitution of the macromolecule can be derived. The ESI-MS technique was recently used for the characterization of different polymers for various purposes such as to determine the accurate molar mass distributions of polymers, to analyze initiating and end-groups of different polymers, to study polymerization mechanisms and kinetics, to determine the initiator efficiency in radical polymerizations, to quantify the efficiency of photoinitiation processes in free radical polymerizations, and to identify the degradation products of different polymers.[4–11] Electrospray ionization is a soft ionization method, which is capable of ionizing soluble polymers. The interfacing of this soft ionization method ESI-Q-TOF MS with collision-induced dissociation (CID) presents a powerful technique for the detailed structural characterization of polymers.

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Poly(2-oxazoline)s are an interesting class of polymers with a wide range of potential applications because of their utilization as biomaterials, as thermo-responsive materials, or as carrier systems for active agents in drug delivery.[12–16] Moreover, they allow an easy access to defined amphiphilic structures for self-assembly processes. The polymerization of oxazolines was first reported in the 1960s by different research groups.[17–20] In this period, living cationic ring-opening polymerization has been established as an important method for the synthesis of well-defined poly-(2-oxazoline)s with narrow molar mass distributions.[21–24] The polymerization of 2-oxazolines exhibits a controlled character and proceeds in the absence of chain transfer and termination reactions at low to medium degrees of polymerization. As a consequence, this method allows the precise synthesis of polymers with well-defined structures, functionalities, topologies, and molar mass distributions, which make poly(2-oxazoline)s suitable for many applications. Besides the synthetic aspects, it is essential to characterize these polymers in a detailed way with different methods to gain information about their molar masses and molar mass distributions as well as about potential side reactions. In the present study, an ESI-Q-TOF MS instrument has been used to elucidate the molar mass distributions of six different poly(2-oxazoline)s, which were synthesized by using microwave irradiation as heating source.[25–26] Additionally, tandem MS analysis provided insights into the fragmentation behavior of these poly(2-oxazoline)s synthesized with different side groups, i.e. methyl, ethyl, 1-ethylpentyl, phenyl, *o*-difluorophenyl, and *p*-*tert*-butylphenyl. The main focus of this work is the investigation of the structural information from the absolute mass values, the elucidation of fragmentation mechanisms with tandem mass spectrometry, and the comparison of the number-average ($\overline{M}_n$) and the weight-average ($\overline{M}_w$) molar masses, and the polydispersity index (PDI) values from ESI-Q-TOF MS with those obtained from size exclusion chromatography (SEC).

Experimental Part

Materials

2-Methyl-2-oxazoline, 2-ethyl-2-oxazoline, 2-phenyl-2-oxazoline, and methyl tosylate were obtained from Acros and distilled to dryness over barium oxide (BaO) and stored under argon. The synthesis procedure of the other monomers was based on synthesis routes that were recently reported in the literature.[27–29] All solvents used for the ESI-MS studies were LC-MS grade, purchased from Sigma Aldrich, and used as received.

General Methods and Instrumentation

The polymerizations were performed under microwave irradiation with temperature control in a Biotage Emrys Liberator mono-mode microwave synthesizer equipped with a noninvasive IR sensor (accuracy: ±2%). The microwave vials were heated to 110 °C overnight, allowed to cool to room temperature and filled with argon before usage. SEC measurements were performed on a Shimadzu system equipped with a SCL-10A system controller, a LC-10AD pump, a RID-10A refractive index detector, a SPD-10A UV-detector at 254 nm, and a PLgel 5 μm Mixed-D column at 50 °C utilizing a chloroform:triethylamine:2-propanol (94:4:2) mixture as eluent at a flow rate of 1 mL · min^{-1}. Molar masses were calculated against polystyrene standards.

Microwave-Assisted Homopolymerization

For each homopolymer, a stock solution containing initiator (methyl tosylate), monomer, and solvent (acetonitrile) was prepared. The monomer concentration was ranging from 0.5 to 1 M with a monomer to initiator ratio of 10 to 20, yielding a molar mass of about 2000 g · mol^{-1}. The vials were heated to 140 °C for a predetermined time in the microwave synthesizer. After cooling, the reaction was quenched by the addition of 50 μL water. Gas chromatography (GC) and SEC samples were prepared to determine the monomer conversion and the molar masses of the polymers, respectively. For the calculation of the monomer conversion, the polymerization solvent was used as internal standard.

ESI-Q-TOF MS Measurements

ESI-Q-TOF MS measurements were performed with a micrOTOF Q-II (Bruker Daltonics) mass spectrometer equipped with an automatic syringe pump from KD Scientific for sample injection. The ESI-Q-TOF mass spectrometer was running at 4.5 kV at a desolvation temperature of 180 °C. The mass spectrometer was operating in the positive ion mode. For the MS/MS mode argon was used as a collision gas. The standard electrospray ion (ESI) source was used to generate the ions. Sample concentrations ranging from 1 to 10 μg · mL^{-1} were injected using a constant flow (3 μL · min^{-1}). The solvent was a chloroform/acetonitrile mixture. There was no salt addition prior to analysis, but ionization occurred readily from the sodium content that is naturally present in the glass. The ESI-Q-TOF MS instrument was calibrated in the range m/z = 50–3 000 using an internal calibration standard (Tunemix solution) which is supplied from Agilent. Data were processed via Bruker Data Analysis software version 4.0.

Results and Discussion

The polymerizations of different 2-oxazolines have been performed using the microwave-assisted living cationic ring-opening polymerization. In the present study, six different methyl initiated poly(2-oxazoline)s with hydroxyl end-groups were synthesized with different side groups such as methyl, ethyl, 1-ethylpentyl, phenyl, *o*-difluoro-phenyl and *p*-*tert*-butylphenyl, respectively. All the poly-(2-oxazoline)s {poly(2-methyl-2-oxazoline) [p(MeOx)], poly-(2-ethyl-2-oxazoline) p(EtOx), poly[2-(1-ethylpentyl)-2-oxa-zoline] [p(EPOx)], poly(2-phenyl-2-oxazoline) [p(PhOx)],

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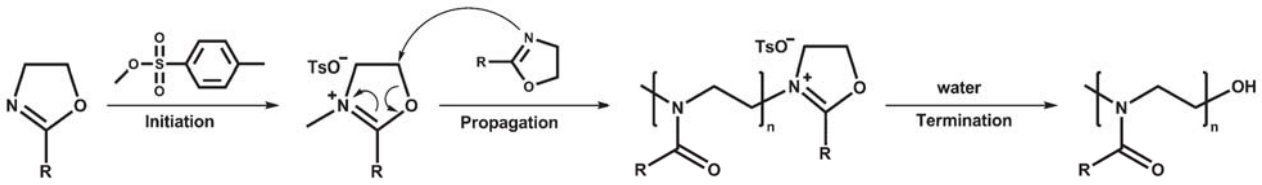

Scheme 1. Schematic representation of the cationic ring-opening polymerization of poly(2-oxazoline)s initiated by methyl tosylate and terminated with water (R: methyl, ethyl, ethylpentyl, phenyl, difluorophenyl and *p-tert*-butylphenyl group).

poly[2-(2,6-difluorophenyl)-2-oxazoline] [p(ODFOx)], and poly(2-*p-ter*butylphenyl-2-oxazoline) [p(*p*TBPhOx)]} were prepared using methyl tosylate as initiator and water as end-capping agent. A schematic representation of the cationic ring-opening polymerization of poly(2-oxazoline)s is depicted in Scheme 1. All polymers were characterized by SEC to reveal $\overline{M}_n$, $\overline{M}_w$, and PDI values and to confirm the living character of the polymerization. ESI-Q-TOF MS measurements were conducted to provide information about the average molar masses of these poly(2-oxazoline)s and to determine the end groups. All spectra were measured in the positive ion mode with mass accuracies in the ppm region. The comparison of the theoretically calculated and the measured isotopic patterns has been made to verify the structure of the polymers. The PolyTools 1.0 software has been used to calculate the average molar masses, the PDI values, the end-group mass, and the repeating unit masses from the ESI-Q-TOF MS measurements by deconvoluting the acquired mass spectra. Table 1 shows the comparison of the $\overline{M}_n$, $\overline{M}_w$, and PDI values from the ESI-Q-TOF MS with those obtained by SEC. The results obtained by the ESI-Q-TOF MS experiments are in a good agreement with the results based on the SEC analysis. However, there are some influences on both techniques which might affect the results; therefore it is complicated to make a clear comparison between these techniques. SEC data can be influenced by the structure of the side groups on different poly(2-oxazoline)s, which may change the hydrodynamic volume of poly(2-oxazoline)s in the SEC solvent. Moreover, correct calibration standards are missing for new polymers. For ESI-Q-TOF MS, ionization problems can be observed due to the mass discrimination effect when the polymer's PDI value is relatively high (PDI > 1.3) and especially for high molar mass polymers. Various poly(2-oxazoline)s with molar mass around 2 000 $g \cdot mol^{-1}$ have been prepared in order to not encounter this kind of problems with ESI-Q-TOF MS. Therefore, for this study, ESI-Q-TOF MS can provide better results compared to SEC within this molar mass range, because it is not affected by any kind of problem within the studied molar mass range.

A broad mass range with high mass accuracy can be investigated by ESI-Q-TOF MS for poly(2-oxazoline)s due to the combination of a quadrupole and an orthogonal acceleration TOF analyzer. This fact is illustrated in Table 2 for the analysis of poly(2-oxazoline)s; mass accuracies of less than 10 ppm were found.

The ESI-Q-TOF mass spectra of the six different poly(2-oxazoline)s are presented in Figure 1. All mass spectra show the expected signal spacings for the respective monomers of the investigated poly(2-oxazoline)s [$\Delta(m/z) = 85.05$ for 2-methyl-2-oxazoline, 99.06 for 2-ethyl-2-oxazoline, 169.14 for 2-(1-ethylpentyl)-2-oxazoline, 147.06 for 2-phenyl-2-oxazoline, 183.05 for 2-(2,6-difluorophenyl-2-oxazoline), and 203.13 for 2-*p-tert*-butylphenyl-2-oxazoline]. The expected polymer chains with methyl as starting group and hydroxyl as end group were observed as the main

Table 1. Number-average molar masses ($\overline{M}_n$), weight-average molar masses ($\overline{M}_w$), and polydispersity indices (PDI) of the poly(2-oxazoline)s with different side groups obtained by SEC and ESI-Q-TOF MS measurements.

Sample	$\overline{M}_{n,\,SEC}$ / $g \cdot mol^{-1}$	$\overline{M}_{w,\,SEC}$ / $g \cdot mol^{-1}$	PDI_{SEC}	$\overline{M}_{n,ESI}$ / $g \cdot mol^{-1}$	$\overline{M}_{w,ESI}$ / $g \cdot mol^{-1}$	PDI_{ESI}
p(MeOx)	2 180	2 420	1.11	2 160	2 232	1.03
p(EtOX)	2 450	2 640	1.08	2 406	2 478	1.03
p(EPOx)	1 730	1 970	1.14	1 464	1 672	1.14
p(PhOx)	1 540	1 750	1.14	1 318	1 492	1.13
p(ODFOx)	2 130	2 550	1.20	1 436	1 779	1.24
p(*p*TBPhOx)	1 950	2 240	1.15	1 632	1 984	1.22

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Table 2. Theoretical and experimental *m/z* ratios of the different poly(2-oxazoline) species obtained by ESI-Q-TOF MS showing deviation from the predicted values.

Sample	$M_{m/z,\ theo}$ [b]	$M_{m/z,\ exp}$ [c]	$\Delta_{m/z}$ [d]
			ppm
$^{Na}p(MeOx)_{20}$ [a]	1 756.0707	1 756.0605	−5.80
$^{Na}p(EtOX)_{20}$ [a]	2 036.3837	2 036.3817	−0.98
$^{H}p(EPOx)_{10}$ [a]	1 724.5001	1 724.4829	−9.97
$^{H}p(PhOx)_{10}$ [a]	1 503.7176	1 503.7089	−5.78
$^{H}p(ODFOx)_{10}$ [a]	1 863.5292	1 863.5133	−8.53
$^{H}p(p\mathrm{TBPhOx})_{5}$ [a]	1 048.6886	1 048.6792	−8.96

[a] The numbers indicate the number of repeating unit of the calculated polymer; Na or H symbols indicate the type of cationization; [b] Calculated monoisotopic masses for the poly-(2-oxazoline)s; [c] Experimental monoisotopic masses for the poly(2-oxazoline)s obtained by the ESI-Q-TOF MS measurements; [d] Mass measurement accuracies as ppm error that was calculated from the difference between the calculated and the experimental monoisotopic masses.

distribution in the ESI-Q-TOF mass spectra; (abbreviated as A in Figure 2 and 3). The second distribution can be explained by the already known chain-transfer reactions that take place during the polymerization of 2-oxazolines leading to hydrogen initiated chains (abbreviated as B in Figure 2 and 3), which are present in the ESI-Q-TOF mass spectra to a lesser extent. In some cases, multiple charged species were also observed extending the molar mass range that can be analyzed.

For the ESI-Q-TOF tandem MS (MS/MS) investigations, all ions with good signal intensities within the main distributions were selected as precursor ions for each poly(2-oxazoline) in order to investigate the effect of the molar masses on the fragmentation mechanisms. Special care was taken to identify the precursor ions which are not coincidentally fitting in the same isolation width area with multiple charged species. The comparison between the different precursor ions shows the same fragmentation series in the ESI-Q-TOF tandem MS analysis. There were no differences for different precursor ions for the studied poly(2-oxazoline)s.

Two representative poly(2-oxazoline)s were selected to show the details of the ESI tandem MS analysis. The first one was poly[2-(1-ethylpentyl)-2-oxazoline] [p(EPOx)] as a representative example for the alkyl substituted poly(2-oxazoline)s. Figure 2 shows the detailed analysis of the p(EPOx) by ESI-MS and MS/MS. In Figure 2a, the ESI-MS data are presented showing an inset with the comparison of the calculated and the experimental isotope patterns for the p(EPOx) with 10 repeating units $[CH_3(NCOC_7H_{15}CH_2CH_2)_{10}OH + H^+]$, which was also selected as a parent peak for tandem MS analysis to investigate the fragmentation patterns. Figure 2b shows the zoom in between $m/z = 1\,705$ and 1 755; it can be seen that there are four distributions which correlate to two main distributions (CH_3- and H− as initiating groups) formed via different cationization processes (Na^+ and H^+). The results of the ESI-Q-TOF MS/MS analysis of p(EPOx) can be seen in Figure 2c, which shows different fragmentation series that are explained in Scheme 2, and the *m/z* difference between two sequential fragmentation ions within one series corresponds to $m/z = 169.14$ units representing the mass of one repeating unit of p(EPOx).

Lattimer and coworkers proposed a 1,4-hydrogen and an ethene elimination mechanisms for poly(ethylene glycol)s, in which the fragmentation products were formed by the elimination of H_2 or C_2H_4 from the backbone by using the fast-atom bombardment (FAB) ionization method.[30–32] Poly(2-substituted-2-oxazoline)s have the same ethene spacing group between the heteroatoms as poly(ethylene glycol)s. Adaptation of the 1,4-hydrogen and the ethene elimination mechanisms to the poly(2-ethyl-2-oxazoline)s have been previously discussed for MALDI-TOF MS/MS measurements,[33] and are presented in this study as well. As shown in Scheme 2a and b, there are two possible rearrangements of the six-membered transition state resulting in the double series A (+) and A′ (×) obtained by ethene elimination and B (•) and B′ (■) obtained from hydrogen elimination, respectively. X and X' species formed via 1,4-hydrogen and ethene elimination were not observed in the ESI-MS/MS spectra. This situation can be explained by further fragmentation of these species to D (▼) and D′ with alkyl elimination on the side chain of X and X′, which is explained in Scheme 2d. The signal intensities of serie D′ are marginal, as a result they have not been highlighted in Figure 2c, d, and e.

Another possible mechanistic explanation for the fragmentation products obtained via ESI-Q-TOF MS/MS is the McLafferty rearrangement[34] as demonstrated in Scheme 2 (c). A (+), A′ (×) and B (•), B′ (■) can also be formed with this kind of rearrangement. McLafferty rearrangement is a β-cleavage with an accompanying transfer of a γ-hydrogen (hydrogen atom in the γ-position to an unsaturated functional group); a scission can take place with the aid of the side group to form a double bond end group. This mechanism is irrespective of the position of the charge. The McLafferty rearrangement has also been postulated in the previously carried out MALDI-TOF-MS/MS study of poly(2-ethyl-2-oxazoline)s.[33] This mechanism also explains the absence of X and X′ species, which were proposed via an elimination mechanism. As a result, it can be concluded that the McLafferty rearrangement is the preferred fragmentation mechanism for these polymers;

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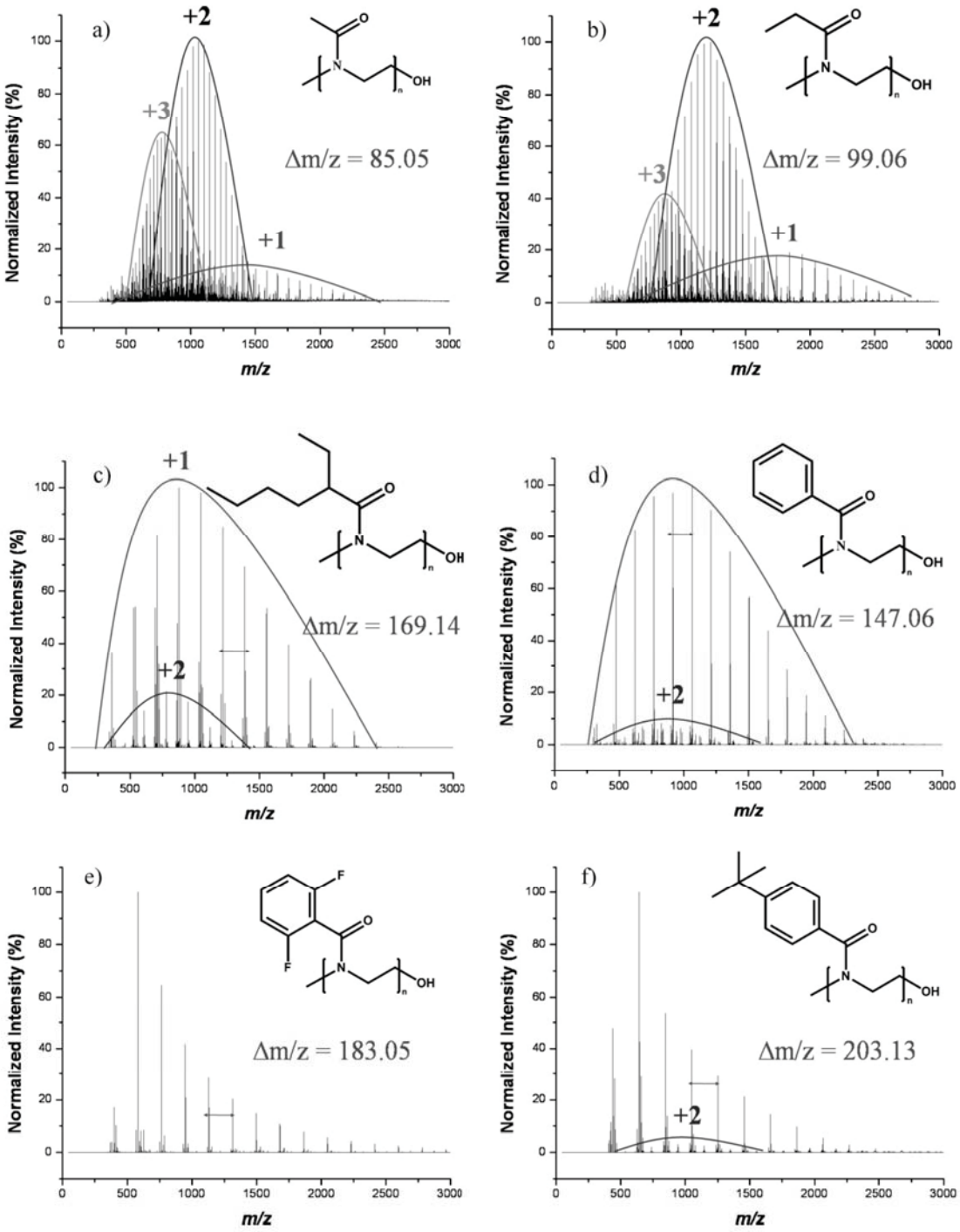

Figure 1. ESI-Q-TOF mass spectra of poly(2-oxazoline)s with different side groups: (a) methyl (MeOx), (b) ethyl (EtOx), (c) ethylpentyl (EPOx), (d) phenyl (PhOx), (e) 2,6-difluorophenyl (ODFOx), and (f) *p-tert*-butylphenyl oxazoline (pTBPhOx). Different lines indicate the singly, doubly, and triply charged molar mass distributions (noted as +1, +2, and +3).

however, the McLafferty rearrangement alone is not sufficient to explain all the other fragmentation products.

Side group elimination occurs in the beginning of the fragmentation of poly(2-alkyl-2-oxazoline)s forming W (▲), the proposed mechanism is demonstrated in Scheme 2e. However, this mechanism was not observed for poly(2-aryl-2-oxazoline)s.

In Scheme 2f, following the cleavage of the hydroxyl group with one monomer unit, the more stable cationic oxazolinium species can be formed and the depolymeriza-

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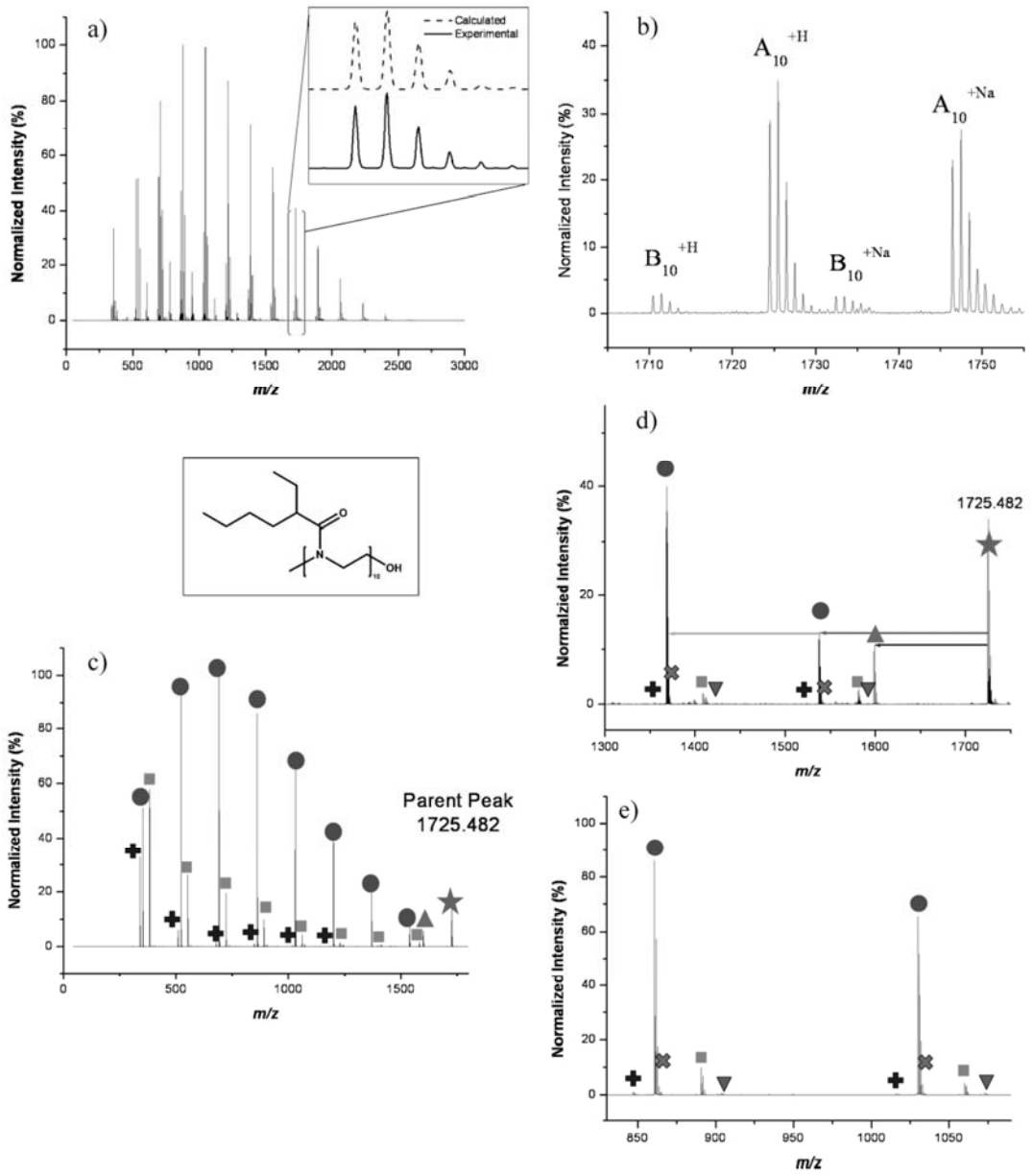

Figure 2. (a) ESI-Q-TOF mass spectrum of p(EPOx), the inset represents a zoom into the isotopic pattern of the selected parent ion for MS/MS and the calculated isotope pattern for this structure, (b) zoom into the ESI-Q-TOF mass spectrum of p(EPOx) in the range between *m/z* 1705 and 1755, (c) ESI-MS/MS spectrum of the parent peak with 10 repeating units marked with ★ and the structure of investigated poly-(2-oxazoline), (d) zoom into the MS/MS spectrum below the parent peak with marked observed fragmentation series, (e) zoom into the MS/MS spectrum in the range between *m/z* = 825 and 1 090 with the observed fragmentation series marked.

tion of the monomer can be followed down to one monomer repeating unit with the starting group. This kind of fragmentation might occur via a depolymerization mechanism, which is similar to the polymerization mechanism of 2-substituted-2-oxazolines. This mechanism can explain the high intensity of the B (•) species in the ESI MS/MS spectrum. An additional proof supporting this suggested mechanism was obtained by the following experiments: when the sodiated parent peak was selected for tandem MS analysis all the fragmentation products had similar intensities in the ESI MS/MS spectrum; when the protonated parent peak was selected there was a high intensity of the B (•) species which might occur from depolymerization processes.

The second polymer selected was poly(2-phenyl-2-oxazoline) p(PhOx) as a representative example of aryl-substituted poly(2-oxazoline)s. Figure 3a shows the ESI-MS data, and the inset exhibits the comparison

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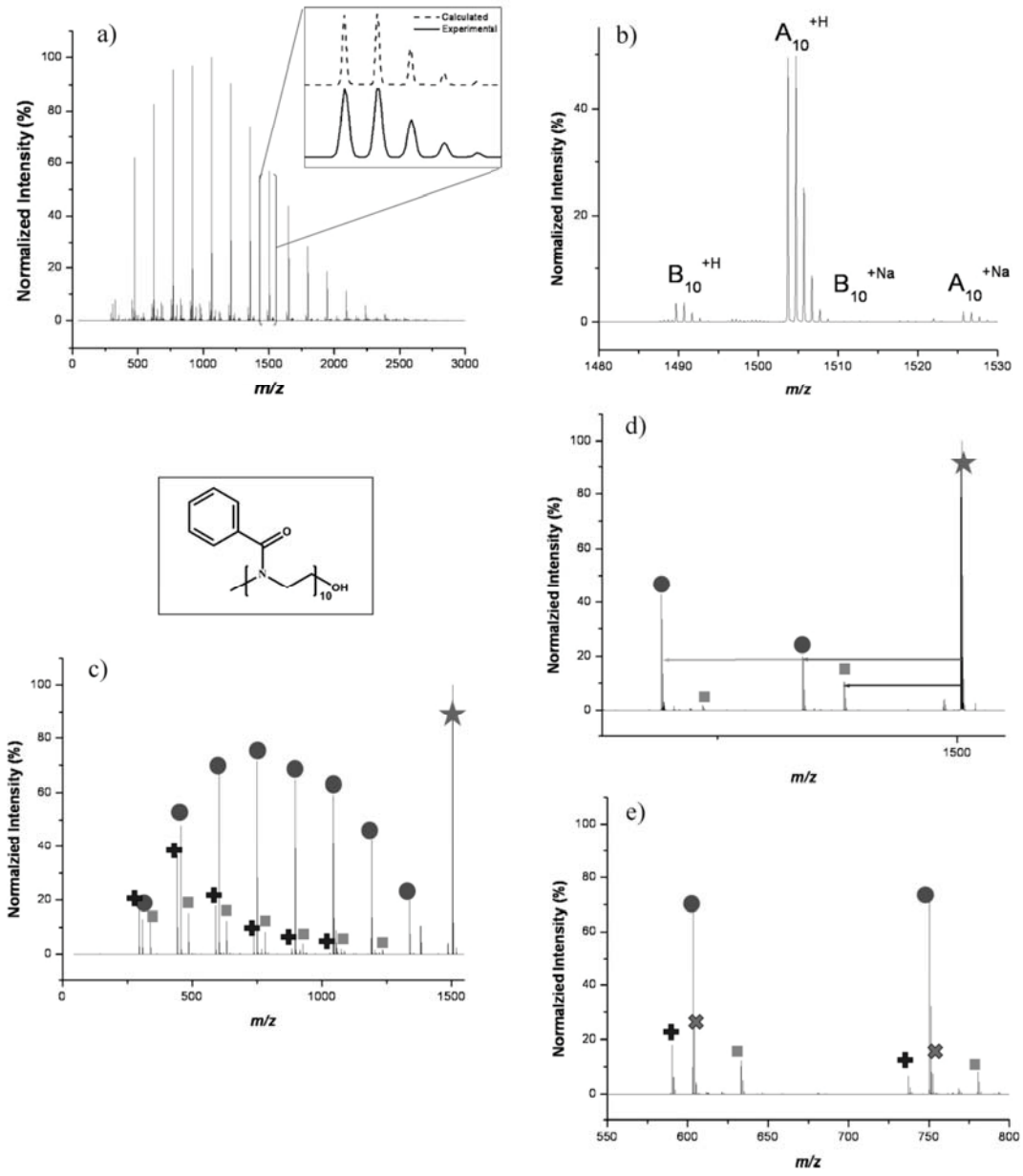

Figure 3. (a) ESI-Q-TOF mass spectrum of p(PhOx), the inset represents a zoom into the isotopic pattern of the selected parent ion for MS/MS and calculated isotope pattern for this structure, (b) zoom into the ESI-Q-TOF mass spectrum of p(PhOx) in the range between $m/z = 1\,480$ and 1 530, (c) ESI-MS/MS spectrum of the parent peak with 10 repeating units marked with ★ and the structure of investigated poly(2-oxazoline), (d) zoom into the MS/MS spectrum below the parent peak with marked observed fragmentation series, (e) zoom into the MS/MS spectrum in the range between $m/z = 550$ and 800 with the observed fragmentation series marked.

between the calculated and the experimental isotope patterns for the p(PhOx) with 10 repeating units $[CH_3(NCOC_7H_5CH_2CH_2)_{10}OH + H^+]$, which was also selected as a parent peak to explain the fragmentation patterns. Figure 3b shows the zoom in between $m/z = 1\,480$ to 1 530, where four distributions, which are actually two main distributions (CH_3- and $H-$ as starting groups), formed via different cationization processes (Na^+ and H^+). The ESI-Q-TOF MS/MS analysis of p(PhOx) can be seen in Figure 3c, which displays different fragmentation series, and the mass difference between these series was 147.06 m/z units representing the mass of one repeating unit of p(PhOx).

The ESI-Q-TOF MS/MS analysis of different poly-(2-substituted-2-oxazoline)s revealed that the polymers with an aryl side chain showed no fragmentation within the side group, thus the W (▲) species were not observed for poly(2-aryl-2-oxazoline)s, which relates to the slower

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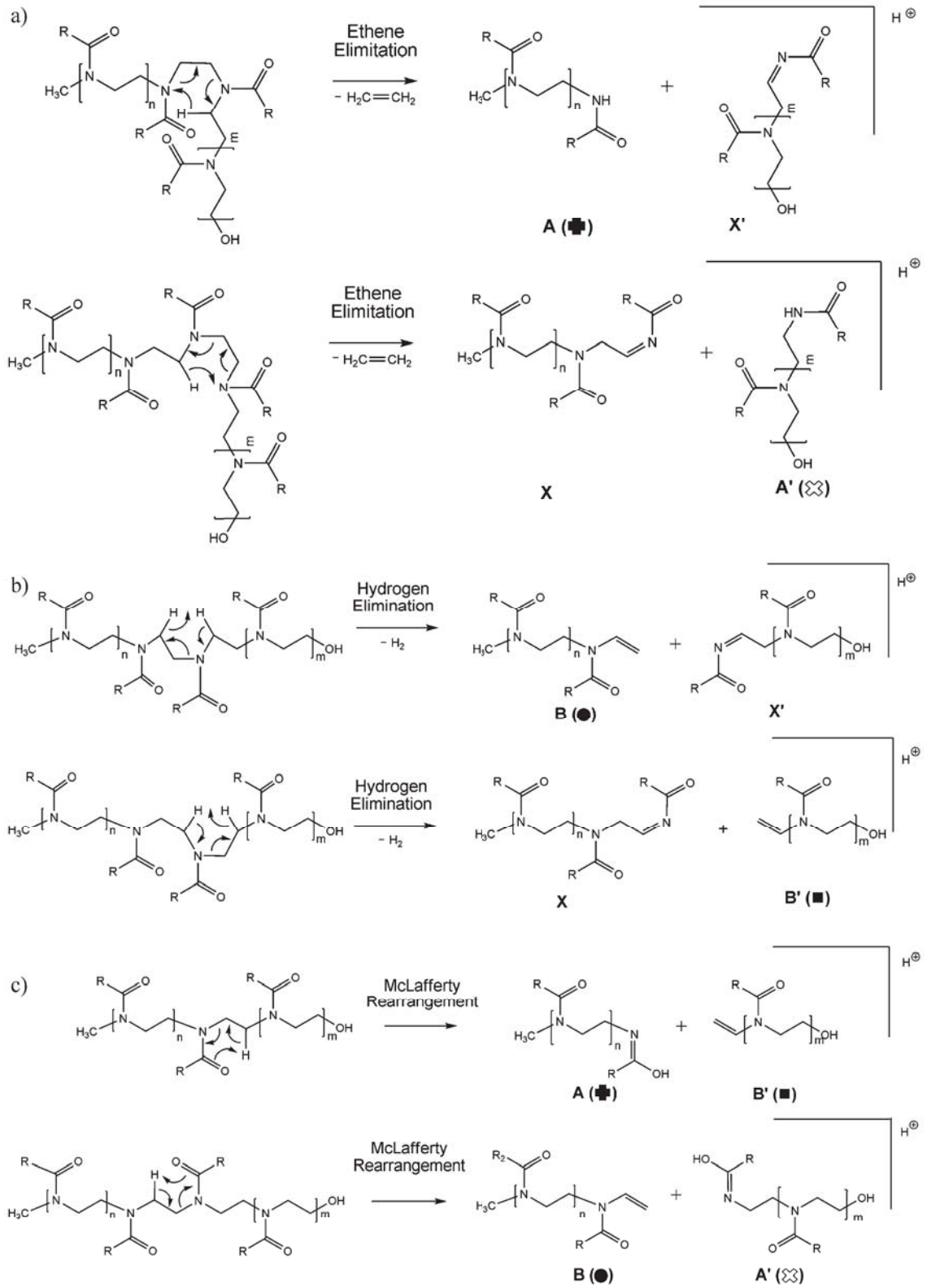

Scheme 2. Schematic representation of the suggested fragmentation mechanisms: (a) ethene elimination forming A and A′ series, (b) hydrogen elimination forming B and B′ series, (c) McLafferty rearrangement forming A, A′ and B, B′ series, (d) alkyl elimination on the side chain (shown for poly(2-(1-ethylpentyl)-2-oxazoline)s) forming D and D′ series, (e) side group elimination for poly(2-alkyl-2-oxazoline)s, and (f) suggested depolymerization mechanism.

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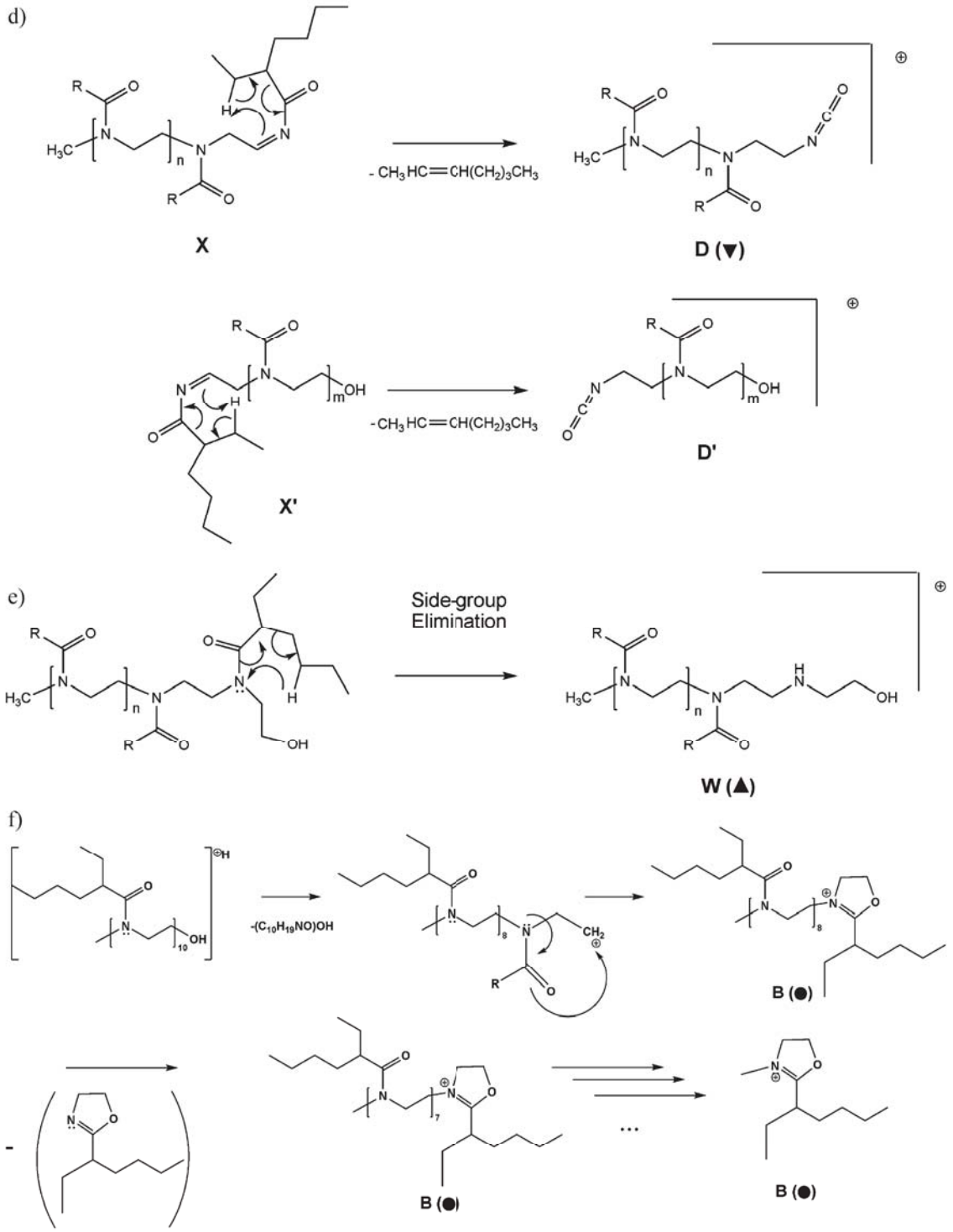

Scheme 2. (*Continued*)

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hydrolysis rate of p(PhOx) compared to p(MeOx) as recently reported.[35] This represents the first observed difference between poly(2-alkyl-2-oxazoline)s and poly(2-aryl-2-oxazoline)s in tandem MS analysis. The second difference was that the D (▼) and D′ species were not observed in the ESI-MS/MS analysis of poly(2-aryl-2-oxazoline)s which were obtained via alkyl elimination on the side group for poly(2-alkyl-2-oxazoline)s. A McLafferty rearrangement was again the possible fragmentation mechanism as suggested earlier for poly(2-alkyl-2-oxazoline)s, which was also proposed for the obtained fragmentation products from poly(2-aryl-2-oxazoline)s [A (+), A′ (×), B (•), and B′ (■)].

Conclusion

In this contribution, the successful characterization of six different poly(2-oxazoline)s with various side groups were performed for the first time via ESI-Q-TOF MS. Utilizing the results of the mass spectrometry investigations, the fragmentation mechanisms for these poly(2-oxazoline)s with different side groups and their differences have been discussed in detailed. ESI-Q-TOF MS has been shown to be a very useful technique for the analysis of macromolecular structures of poly(2-oxazoline)s with different side groups. Possible fragmentation mechanisms such as the 1,4-hydrogen and the ethene elimination, as well as the McLafferty rearrangement, were proposed for the obtained fragmentation products. Side group elimination in the beginning and alkyl elimination on the side chain were only observed for poly(2-alkyl-2-oxazoline)s, these side group related fragmentation mechanisms were not observed for poly(2-aryl-2-oxazoline)s, whereby it should be noted that this was generally the case for the relatively limited library space investigated here. Overall, it can be concluded from this study that the usage of ESI-MS and ESI-MS/MS is very useful for the determination of the chemical structure of poly(2-oxazoline)s and the identification of its initiating and end-groups.

The information gained from this study will also help to build a tandem MS product ion library of poly(2-oxazoline)s with different side groups including fragmentation pathways which will provide necessary knowledge for the future to make a fast and automated identification of these polymers possible.

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Publication P3:

Determination of the relative ligand binding strengths in heteroleptic Ir^{III} complexes by ESI-Q-TOF tandem mass spectrometry

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Esra Altuntaş, Andreas Winter, Anja Baumgärtel, Renzo M. Paulus, Christoph Ulbricht, Anna C. Crecelius, Nikolaus Risch, and Ulrich S. Schubert

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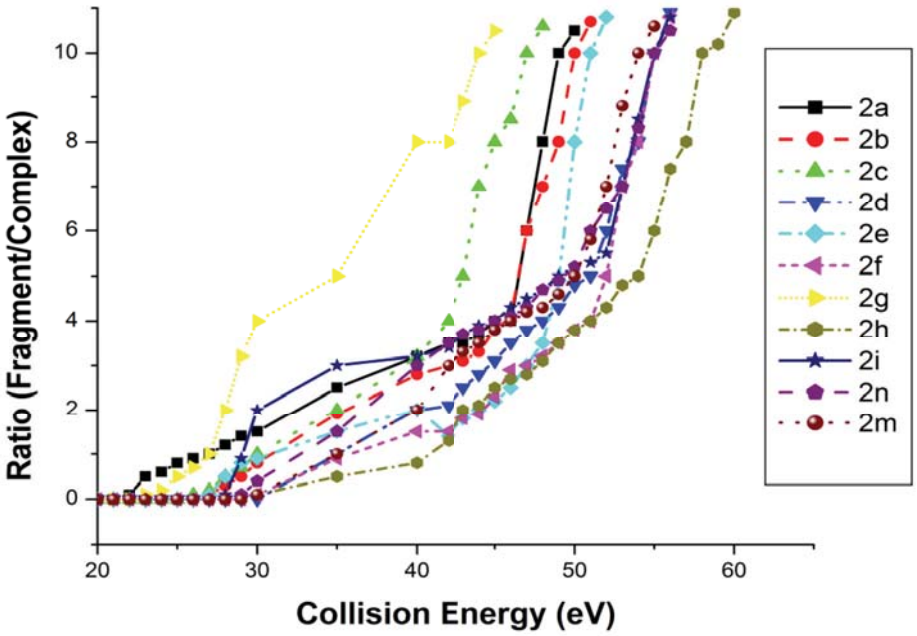

Research Article

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Determination of the relative ligand-binding strengths in heteroleptic Ir^{III} complexes by ESI-Q-TOF tandem mass spectrometry

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An electrospray ionization quadrupole time-of-flight mass spectrometer has been utilized to investigate the relative ligand-binding strengths in a series of heteroleptic-charged iridium(III) complexes of the general formula $[(C^{\wedge}N)_2Ir^{III}(S\text{-tpy})](PF_6)$ by using variable collision energies. Collision-induced dissociation experiments were performed in order to study the stability of the Ir^{III} complexes that are, for instance, suitable phosphors in light-emitting electrochemical cells. The ratio of signal intensities belonging to the fragment and the undissociated complex depends on the collision energy applied for the tandem mass spectra (MS/MS) analysis. By defining the threshold collision energy and the point of complete complex dissociation, it is possible to estimate the relative complex stabilities depending on the nature of the coordinated ligands [i.e. type of cyclometalating ligand (C^N), substituents on the S-shaped terpyridine (S-tpy)]. The collision energy values differed as a function of the coordination sphere of the Ir^{III} centers. Copyright © 2011 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: ESI MS; tandem mass spectrometry; relative binding strength; iridium(III) complexes

INTRODUCTION

Because of their promising photophysical behavior, ionic character and good solubility in polar organic solvents or even in aqueous media, cationic iridium(III) complexes have gained much interest in recent years;[1] applications as emissive materials in light-emitting electrochemical cells (LECs),[2–4] biolabeling/bioimaging,[5,6] sensing[7,8] as well as photocatalysis[9,10] have been established. The Neve group introduced a synthetic protocol that is today the most commonly used concept to synthesize cationic *bis*-cyclometalated Ir^{III} polypyridyl complexes.[11,12] Using this procedure, a broad range of 2,2′-bipyridine, 1,10-phenanthroline and 2,2′:6′,2″-terpyridine derivatives has found use as neutral bidendate ligands.[1,5,13–16] Recently, also 2-(1,2,3-1H-triazol-4-yl)-pyridine units have been utilized as bidentate ancillary ligands.[17,18]

It has been demonstrated that the introduction of either electron-withdrawing or electron-donating groups on the cyclometallating ligands in combination with lateral (π-conjugated) substituents on the polypyridyl ligand enables the alteration of the electro-optical properties of the complexes.[5] For instance, Bernhard and co-workers prepared a library of luminophores featuring high color versatility, a broad range of excited-state lifetimes (i.e. nanoseconds to several microseconds) as well as remarkable photoluminescence quantum yields; these complexes featured different substituents [e.g. -F, $-CF_3$, $-C(CH_3)_3$] on the cyclometalating ligands and various types of monodentate or bidentate ancillary ligands.[19,20] In neutral (e.g. *tris*-cyclometalated) complexes, the ligand field stabilization energy strongly depends on the position of the substituents with respect to the cyclometalating carbon atom and the same holds for charged complexes.[21–23] The Thompson group was able to show that the excited-state properties of *bis*-cyclometalated Ir^{III} complexes can be chemically controlled simply via the nature of the ancillary ligand.[24] The enhancing effect of the increased steric hindrance of the ancillary polypyridyl ligands on the photoluminescence quantum yield has been reported by Wu and co-workers.[25] Supported by computational studies, Huang and co-workers presented their concept of tunable emission via the expansion of the π-conjugated system of the chelating ligand.[26] Related to this, the groups of Mussini, Roberto and Fantacci showed that the photophysical properties of cationic *bis*-cyclometalated Ir^{III} complexes could be further fine tuned via lateral electron-rich or electron-poor substituents on phenanthroline-type ligands.[27]

A variety of functionalized 2,2′:6′,2″-terpyridine derivatives and related structures have also been employed as the bidentate

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ligand in *bis*-cyclometalated Ir^{III} compounds and enabled the tuning of the photophysical properties.[28,29]

While the interplay of electronic and/or steric properties of ligands – cyclometalating and non-cyclometalating ones – in the coordination sphere of Ir^{III} ions and the optoelectronic properties of the resulting complexes are well understood, the impact of such ligands on the stability of the complexes has only been investigated to a minor extend. In particular, the robustness of luminescent chromophores is a requirement for the development of durable optoelectronic devices. Besides isothermal titration calorimetry,[30,31] differential scanning calorimetry,[30,32,33] and thermogravimetric analysis[34–36] and the modern tools of MS[37,38] have been applied to determine the stability of various types of transition metal ion complexes. With respect to the latter ones, soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) have become available to investigate the strength of non-covalent interactions in the gas phase.[39–42] MALDI time-of-flight (TOF) MS has been widely used for the investigation of non-covalent interactions such as complexes of transition metal ions with peptides and metal ligand complexes.[43–45] The most important advantage of MALDI MS is the wide accessible mass range, which makes this technique ideal for the analysis of large metallo-supramolecular assemblies and macromolecules. However, this technique has a major disadvantage when analyzing small organic or organometallic compounds due to the formation of matrix adducts. Thus, misleading fragments and/or adducts can be produced by this method; whereas this is generally not the case when ESI MS is applied. ESI MS is one of the most important methods to elucidate the composition of metallo-supramolecular structures in solution and can, for instance, be used to investigate metal-to-ligand interactions or to determine metal–ligand binding energies.[46–49] However, ESI MS has also some disadvantages such as cluster formation under mild conditions and a low sensitivity under harsh conditions. These drawbacks can be overcome by adjusting the instrumental parameters, and ESI MS is, therefore, highly suited for the fast and sensitive determination of the relative gas phase stabilities of cationic Ir^{III} complexes.

In this study, the characterization of a library of cationic *bis*-cyclometalated Ir^{III} complexes by ESI-Q-TOF MS and MS/MS is presented. The key target of the study is to elaborate the relative ligand-binding strength in Ir^{III} complexes in the gas phase by ESI-Q-TOF mass spectrometry using variable collision energies (1–200 eV). The ratio of signal intensities arising from the fragment and the undissociated complex depends on the collision energy utilized in the tandem mass spectrometric analysis. By defining the threshold collision energy and the point of complete complex dissociation, one can estimate the complex stabilities in the gas phase in dependence on the coordinated ligand(s).

EXPERIMENTAL

Materials

All solvents used for the ESI-Q-TOF measurements were purchased from Sigma Aldrich and used as received. $IrCl_3 \cdot 3H_2O$ was kindly provided by Haereus. The S-shaped terpyridines (S-tpy) were prepared as reported previously.[28,50,51] The synthesis of the dimeric μ-chloro-bridged Ir^{III} precursor complexes $[(C^{\wedge}N)_2Ir\text{-}\mu\text{-}Cl]_2$ ($C^{\wedge}N$ denotes a cyclometalated ligand) was carried out according to known procedures.[20,52,53]

General procedure for the synthesis of the bis-cyclometalated iridium(III) S-tpy complexes (1–3)

A suspension of the respective Ir^{III} precursor complex $[Ir(C^{\wedge}N)_2\text{-}\mu\text{-}Cl]_2$ (0.038 mmol) in methanol (10 mL) was added to a solution of the S-shaped terpyridine (0.077 mmol) in CH_2Cl_2 (8 mL). After heating under reflux for 3 h, the clear yellow solution was cooled to room temperature, a saturated solution of NH_4PF_6 in methanol (2 mL) was added, and stirring was continued for additional 3 h. Evaporation of the solvents yielded the crude product. For removal of excessive ammonium salt, the complex was redissolved in CH_2Cl_2 (20 mL) and extracted with water (3×20 mL). This was followed by drying over $MgSO_4$, concentration of the solution and precipitation in pentane. The crude products were further purified, when necessary, by flash column chromatography on neutral Al_2O_3 using CH_2Cl_2/acetone (3 : 1 ratio) as eluent.[28]

ESI-Q-TOF MS and MS/MS measurements

The ESI-Q-TOF MS measurements were performed with a micrOTOF-Q II mass spectrometer (Bruker Daltonics) equipped with an automatic syringe pump for sample injection (KD Scientific). The ESI-Q-TOF mass spectrometer was run at 4.5 kV with a desolvation temperature of 180 °C. The mass spectrometer was operating in the positive ion mode; the standard ESI source was used to generate the ions. The concentration of the samples was 10 μg mL^{-1} (acetonitrile as solvent) and all samples were injected using a constant flow rate (3 μL min^{-1}) of sample solution. Elemental composition of the singly charged heteroleptic iridium(III) complexes was confirmed by accurate mass measurements. For the tandem mass spectrometric (MS/MS) measurements, argon was used as the collision gas. The complex ion without counter ion $[M\text{-}PF_6]^+$ was mass selected and activated in the collision-induced dissociation (CID) mode by increasing the collision energies (1–200 eV) up to the energy where the respective complex experienced full dissociation. The ESI-Q-TOF MS instrument was calibrated in the m/z range of 50–3000 using an internal calibration standard (Tunemix solution), which was supplied from Agilent. The collected data were processed via Bruker Data Analysis software (version 4.0).

RESULTS AND DISCUSSION

The abstraction of ligands from the coordination sphere of a transition metal ion can be achieved by various external stimuli, such as pH value or competing ligands (in solution) as well as temperature and redox chemistry. Upon dissociation, metal complexes experience a significant change in their photophysical and electrochemical properties, which is of relevance for their potential application, as photoactive and/or redox-active species, in optoelectronic devices. For instance, cationic *bis*-cyclometalated Ir^{III} complexes find use, as emitters, in LECs.[1] LECs represent an alternative to light-emitting diodes. Crucial issues concerning efficiency limitation, response times, multicolor approaches and device lifetimes were reviewed by Slinker *et al.*[4] For the structural and electronic parameters influencing the stability of cationic *bis*-cyclometalated complexes of d[6] transition metal ions to be elucidated, a library of Ir^{III} complexes $[(C^{\wedge}N)_2Ir(\mathbf{1})](PF_6)$ **2** was synthesized by bridge-splitting reaction of the appropriate chloro-bridged Ir^{III} dimeric precursor complex according to Neve and co-workers (Scheme 1).[54] Thereby, the cyclometalating $C^{\wedge}N$ ligands addressed the metal center both sterically and electronically, whereas the bidentate $N^{\wedge}N$ S-shaped terpyridine ligands **1**

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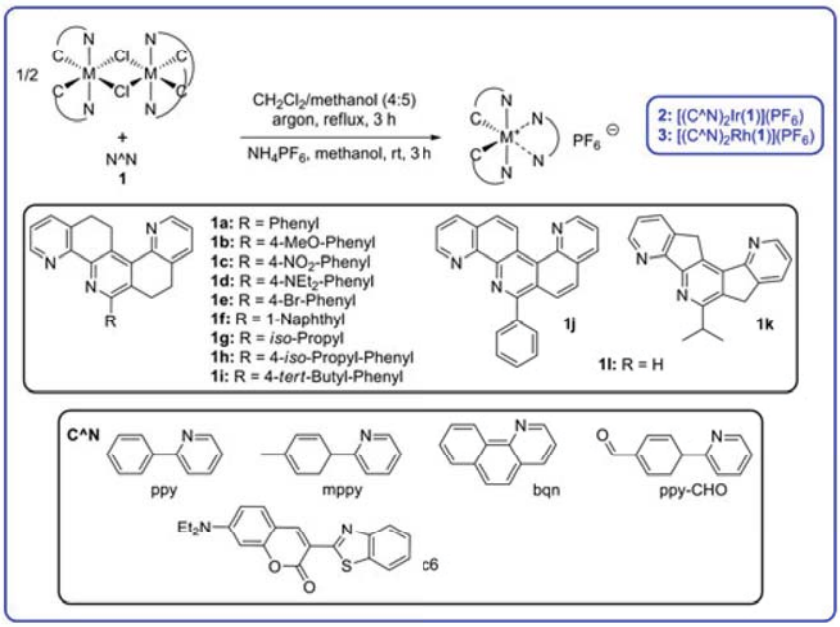

Scheme 1. Schematic representation of the synthesis of the studied heteroleptic iridium(III) (**2**) and rhodium(III) complexes (**3**).

mainly exhibited electronic effects (because of the similar size and shape of the ligands **1**, their steric demand has to be expected within the same order of magnitude). Similarly, the Rh^{III} complexes **3** (Scheme 1) were prepared to investigate if the smaller size and higher charge density of the Rh^{III} center had any influence on the relative stability of the respective complexes.[55]

All complexes were characterized by ^{1}H NMR spectroscopy and elemental analysis, the electro-optical properties of Ir^{III} complexes **2** were already published elsewhere.[28] In the following, the mass spectrometric investigation of **2** and **3** will be discussed in detail. Representatively, the ESI-Q-TOF and MALDI-TOF mass spectra of $[(ppy)_2Ir(\mathbf{1a})](PF_6)$ are depicted in Figure S1 (Supporting Information). The MALDI-TOF mass spectrum revealed significant fragmentation of the complex (via abstraction of the N^N ligand **1a**), even without performing tandem MS analysis. Even kinetically robust non-covalent metal-to-ligand coordinative bonds are known to partially break during the MALDI process due to the applied thermal energy of the laser pulses.[37,38] In contrast, ESI, as by far the softer ionization technique, did not show any considerable fragmentation for most complexes **2** in the MS mode and was, consequently, chosen for the subsequent investigation of the relative binding strength. As an exception, for complexes **2j** and **2k**, fragmentation occurred already in the MS mode, which was attributed to unfavored coordination geometry (due to the highly rigid ligand **1j**) and to a decreased stability of the coordination of **1k** to the Ir^{III} center (due to the expanded bite angle). Moreover, complexes **2o** and **2p** comprising aldehyde-functionalized ppy (ppy-CHO) and coumarin-6 (c6), as cyclometalating ligands, respectively, also showed partial dissociation under the measurement conditions: the former revealed fragmentation into $[(ppy\text{-}CHO)_2Ir]^+$ and $[\mathbf{1a}]^+$, whereas **2o** experienced the cleavage of an ethyl moiety from one c6 ligand. Finally, exhausted cleavage of the unsubstituted S-tpy ligand **1l** (within the detection limit of method) into $[(ppy)_2Ir]^+$ became apparent.

For all complexes **2**, the signal assignments were confirmed by accurate mass measurements and comparison of the obtained m/z values to the theoretical ones. In general, a good correlation between these values was observed with deviations of less than 5 ppm confirming the identity of the investigated species (Table S1).

The ESI-Q-TOF MS/MS spectrum of the heteroleptic Ir^{III} complex **2a** is depicted in Figure S2. The peak at m/z = 862.25 in the ESI-Q-TOF mass spectrum was utilized, as precursor ion, for the subsequent analysis by CID experiments in order to gain insights of the fragmentation behavior of complexes $[(C^{\wedge}N)_2Ir(\mathbf{1})]^+$. The CID technique was developed by the McLucky[56] and Brodbelt groups[48,57] and allows a relative comparison of the bonding parameters between structurally related compounds, in particular for non-covalent systems. The ESI-Q-TOF MS/MS spectrum revealed singly charged ions arising from the loss of either the S-tpy ligand or one cyclometalating ligand. It has to be pointed out that no additional fragmentation step (i.e. the abstraction of a second ligand) could be observed, not even at elevated collision energies. As expected, the connectivity between the ppy ligands and the Ir^{III} center in **2a** with partial covalent character was significantly stronger than the purely non-covalent interaction of the S-tpy and the metal ion. Because complexes **2b-i** only differed from **2a** in the substituent R of the peripheral S-tpy ligand, similar results were obtained under ESI-Q-TOF MS/MS conditions.

The relative binding strengths of the cationic Ir^{III} complexes **2** were estimated by utilizing ESI-Q-TOF MS and MS/MS in the following way:[58] for each complex, three stock solutions with a concentration of 10 μg mL^{-1} of **2** were prepared and measured under same conditions with varying collision energy. The relative signal intensities for the fragment $[(C^{\wedge}N)_2Ir]^+$ and complex $[(C^{\wedge}N)_2Ir(\mathbf{1})]^+$ peaks were taken from the calibrated mass spectra to calculate the $[(C^{\wedge}N)_2Ir]^+/[(C^{\wedge}N)_2Ir(\mathbf{1})]^+$ ratios. This ratio of signal intensities depended on the collision energy used for the tandem mass spectra acquisition (i.e. the collision energy utilized for the CID experiments). In these experiments, the most abundant peak in the resulting spectra – always corresponding to the singly charged cation peaks (i.e. loss of the PF_6^- counter ion) – were selected as precursor ions and were separated according to their masses and accelerated into the collision cell of the ESI-Q-TOF mass spectrometer. The collision energy was varied over a wide range

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(1–200 eV) to identify the threshold collision energy values and the point of complete complex dissociation for each complex. In this study, the threshold collision energy can be considered as the collision energy value sufficient to produce fragments from **2** in the collision cell.[48] At the point of complete complex dissociation, the $[(C^{\wedge}N)_2Ir]^+/[(C^{\wedge}N)_2Ir(\mathbf{1})]^+$ ratio is ≥10 (as defined by Meier *et al.*[37]); by comparing the applied collision energies, one can subsequently conclude on the relative gas phase stability of **2**, as a function of the C^N and S-tpy ligands within the coordination sphere of the Ir^{III} centers.

The ratio of signal intensities of fragment $[(C^{\wedge}N)_2Ir]^+$ and complex $[(C^{\wedge}N)_2Ir(\mathbf{1})]^+$ increased continuously with increasing collision energy utilized for the tandem MS analysis, indicative for the fragmentation/destruction of the complex. Representatively, the fragmentation profile of $[(ppy)_2Ir(\mathbf{1a})]^+$ in the tandem MS process, as a function of the applied collision energy, is depicted in Fig. 1. In each mass spectrum, the signal intensities were normalized to the highest intensity peak. All investigated Ir^{III} complexes revealed the same behavior, however, the point of complete dissociation ($[(C^{\wedge}N)_2Ir]^+/[(C^{\wedge}N)_2Ir(\mathbf{1})]$ ≥10) was reached at different collision energies utilized for the ESI-Q-TOF MS/MS studies. The corresponding degradation curves reflecting this characteristic trend are summarized in Fig. 2: for all complexes, the collision energy is plotted *versus* the $[(C^{\wedge}N)_2Ir]^+/[(C^{\wedge}N)_2Ir(\mathbf{1})]^+$ ratio. Depending on the nature of the coordination sphere (i.e. C^N and S-tpy ligand), the Ir^{III} complexes **2** exhibited a different degradation behavior under the applied collision energies in the tandem MS analysis. The threshold collision energies as well as the complete complex dissociation energies are summarized in Table 1.

For correlation of the determined dissociation energies in the gas phase to the structure of the complex, i.e. with the nature of the ancillary N^N ligand, the polarizabilities (α) of the S-shaped terpyridines **1** were estimated according to the incremental method of Miller (Table 1) [a calculation of the polarizability according to the Clausius–Mossotti equation was not feasible due to the non-existence of some gas phase parameters of **1** (i.e. density and refractive index)].[59,60] The polarizability of the ligands, i.e. their ability to deform the electron cloud for enhanced coordination, is mainly based on the molecules' size and the bond type. This parameter was reported to be a suitable reference point for describing the ligand's properties, taking the influence of electron-donating/electron-withdrawing substituents

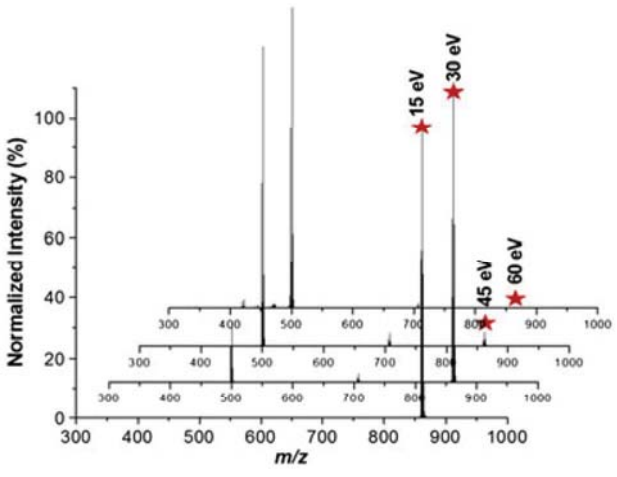

Figure 1. Electrospray ionization quadrupole time-of-flight tandem mass spectra of complex $[(ppy)_2Ir(\mathbf{1a})]^+$ (**2a**) at different collision energies.

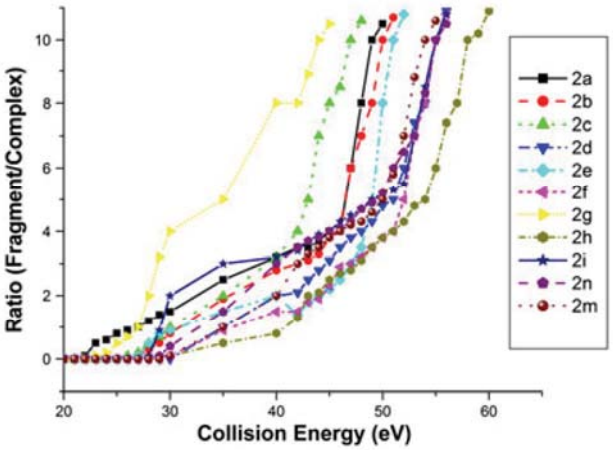

Figure 2. Energy-variable collision-induced dissociation curves for the investigated Ir^{III} complexes **2**.

also into account (however, the actual steric demand of a molecule is not reflected in this model).[48,61,62] The correlation of the calculated polarizabilities with the experimental threshold collision energies is depicted in Fig. 3(a). Because 2-phenyl-pyridine was utilized, as cyclometalating ligand, in all complexes **2a-i** (because of the observed fragmentation in the MS mode, complexes **2j-l** were excluded from this investigation), the differences in complex stability could directly be attributed to the nature of the ligand **1**. In general, the substituent R (Scheme 1) had only a negligible impact on the overall shape and, thereby, on the sterical demand of the S-tpy ligand. Similar results were obtained previously by Satterfield and Brodbelt who investigated the dissociation of substituted phenanthroline complexes by mass spectrometric means.[48] In general, an almost linear increase of the threshold collision energy and, thereby, of the binding energy with increasing polarizability of **1** became apparent. Complex **2f**, comprising the naphthalen-1-yl-substituted ligand **1f**, significantly deviated from this general trend: the highest polarizability within the series should lead to a high stability of the complex with respect to dissociation. However, the considerable steric demand of this particular ligand resulted in unfavorable coordination geometry due to steric interaction with the cyclometalating ligands on the Ir^{III} center.

In a second series, the cyclometalating ligand coordinated to the Ir^{III} center was varied, whereas the S-tpy ligand was kept constant (**1a**). As shown in Fig. 3(b), the polarizability of the C^N ligands[59] also influenced the binding energy of the remote S-tpy ligand: with increasing polarizability, an increase of the binding strength became apparent. Again, two complexes (**2o** and **2p**) had to be excluded from this study because of their fragmentation in the normal MS mode. 7,8-Benzoquinoline (bqn), as cyclometalating ligand, exhibited the highest polarizability within the series and, consequently, the highest dissociation energy was observed for $[(bqn)_2Ir(\mathbf{1a})]^+$ (Table 2).

The relationship between complete dissociation energy and polarizability was shown in Fig. 3(c) and (d). With one exception (complex **2f**), all complexes revealed a good correlation between the complete dissociation energies and polarizability values. There is a linear increase of complete dissociation energies with increasing polarizability values. For Ir^{III} complexes **2** with varying S-tpy ligands, only small differences can be observed, which indicates that the effect of the R substitution is not playing a significant role on binding energies. Nevertheless, it is possible to

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Table 1. Threshold collision energies and the point of complete complex dissociation energies for the investigated Ir^{III} complexes

Complex	Polarizability (10^{-24} cm^3) [a]	Threshold collision energy (eV) [b]	Complete dissociation energy (eV) [c]
2a	42.42	21.0	49.0
2b	44.58	26.0	50.0
2c	42.25	24.5	47.5
2d	50.78	30.0	55.5
2e	44.13	25.5	51.5
2f	59.14	28.5	54.0
2g	38.18	22.0	44.5
2h	47.75	28.5	59.0
2i	49.52	27.5	55.5
2j	42.19	n.a.	46.5
2k	34.63	n.a.	42.5
2l	32.86	n.a.	39.5

[a]Polarizabilities of **1** were estimated via the additive method of Miller.[59]
[b]Threshold collision energy is defined in this study as the minimum collision energy required to initiate fragmentation.[66]
[c]Point of complete complex dissociation is defined as a ratio of $[(C\text{^}N)_2Ir]^+/[(C\text{^}N)_2Ir(\mathbf{1})]^+ \geq 10$.

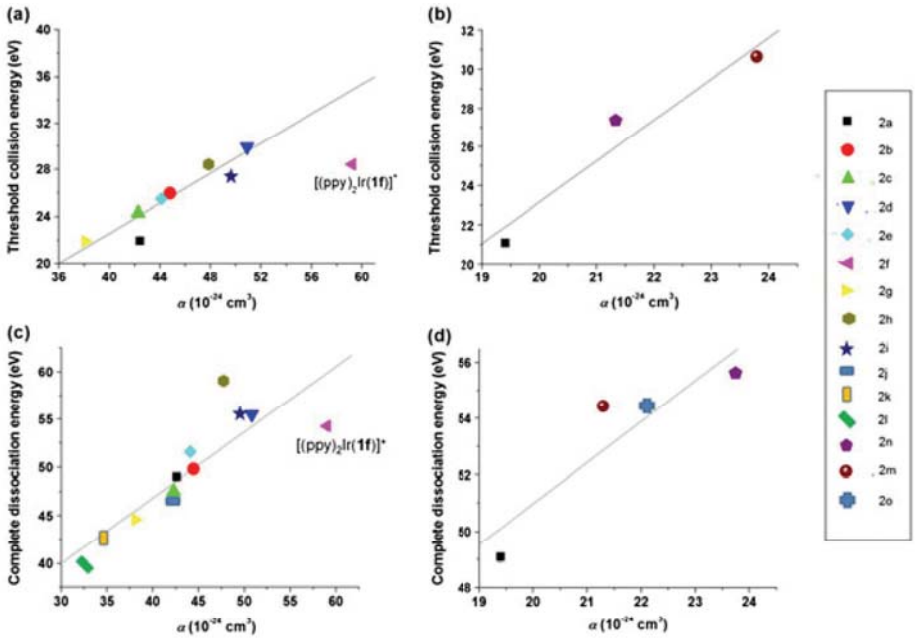

Figure 3. Threshold collision energy *versus* polarizability plot of Ir^{III} complexes **2** with ppy, as cyclometalating ligand (a), with various C^N ligands and **1a** (b) and complete dissociation energy *versus* polarizability plot of Ir^{III} complexes **2** with ppy, as cyclometalating ligand (c), with various C^N ligands and **1a** (d).

Table 2. The threshold collision energies and the point of complete complex dissociation energies for complexes $[(C^N)_2Ir(\mathbf{1a})]^+$

C^N ligand	Polarizability (10^{-24} cm^3) [a]	Complex	Threshold collision energy (eV)	Complete dissociation energy (eV)
ppy	19.39	**2a**: $[(ppy)_2Ir(\mathbf{1a})]^+$	21.0	49.0
mppy	21.31	**2m**: $[(mppy)_2Ir(\mathbf{1a})]^+$	30.5	54.5
bqn	23.79	**2n**: $[(bqn)_2Ir(\mathbf{1a})]^+$	27.5	55.5
ppy-CHO	22.07	**2o**: $[(ppy\text{-}CHO)_2Ir(\mathbf{1a})]^+$	n.a.	54.5
c6	38.34 [b]	**2p**: $[(c6)_2Ir(\mathbf{1a})]^+$	n.a.	57.0

[a]Polarizabilities of the cyclometalating ligands were taken from literature.[66]
[b]Polarizability of c6 was estimated using the incremental method of Miller.[59]

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obtain information about the gas phase stabilities of Ir^{III} complexes **2** from these studies.

The stability of transition metal complexes is not only influenced by the nature of the coordinated ligands (i.e. polarizability and steric demand) but also via the size and the surface charge density of the transition metal ion.[48,63–65] Accordingly, the fragmentation of Ir^{III} complexes **2** under ESI mass spectrometric conditions was compared with that of complexes containing Rh^{III} ions as the lighter group 9 metal ion (because of synthetic reasons, the analogous *bis*-cyclometalated Co^{III} S-tpy complexes could not be investigated). The synthesized Rh^{III} complexes **3** were characterized by high resolution mass spectrometry measurements (Table S2). As expected, complexes **3** exhibited a higher tendency toward cleavage of S-tpy ligand **1**: fragmentation into $[(ppy)_2Rh]^+$ and $[\mathbf{1}]^+$ already occurred in the normal MS mode (Table S3). Thus, the threshold collision energy as indication for the complex stability could not be determined, and the comparison between the homologous complexes **2** and **3** remained qualitative.

CONCLUSION

In this study, the utilization of ESI-Q-TOF tandem mass spectrometry for the determination of the relative ligand-binding strength in a series of cationic *bis*-cyclometalated Ir^{III} complexes of the general type $[(C^{\wedge}N)_2Ir(S\text{-}tpy)](PF_6)$ by using variable collision energy was presented. The charged Ir^{III} complexes revealed different degradation behaviors under the applied collision energies according to the nature of the coordination sphere. The CID spectra showed the preferential loss of the S-shaped terpyridine ligand and the dissociation profile of the complexes induced by increasing the collision energy. The polarizabilities of the S-shaped terpyridines were estimated to correlate the determined gas phase dissociation energies with the structural features of the complexes. A linear increase of the relative binding energies with increasing polarizability of the S-tpy ligands was observed. Thus, ESI MS/MS proved to be a valuable tool for studying the complexational behavior of a large variety of heteroleptic Ir^{III} complexes in the gas phase. These findings demonstrate the usefulness of ESI tandem mass spectrometry to probe the gas phase stabilities of *bis*-cyclometalated Ir^{III} complexes. The results obtained from this study will help to design new stable complexes for different applications (e.g. in LECs), and this method is applicable for the rapid determination of the relative binding strength of heteroleptic Ir^{III} complexes.

Acknowledgements

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Supporting Information

Supporting information may be found in the online version of this article.

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Publication P4:

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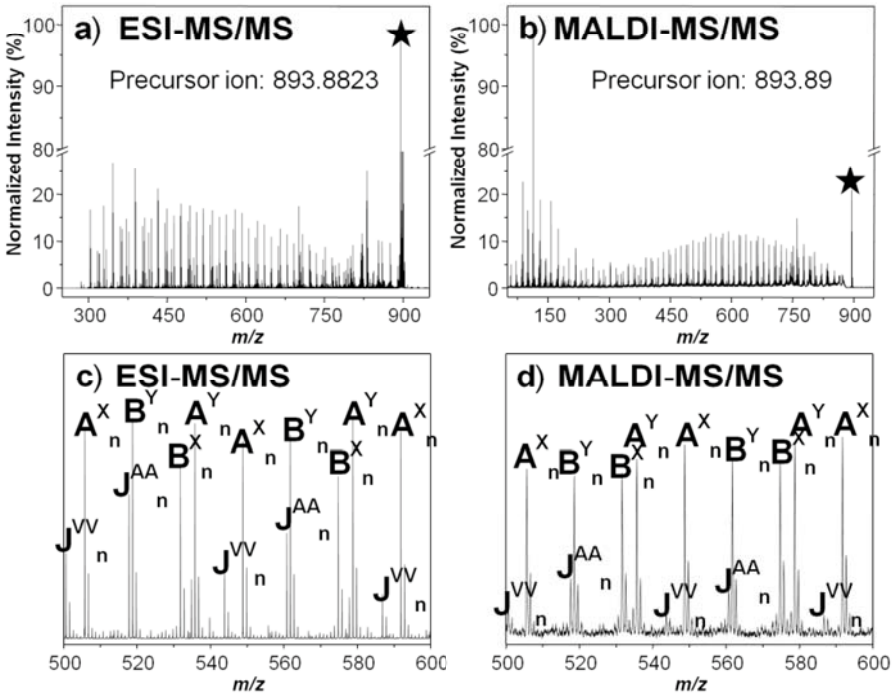

Research Article

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Tandem mass spectrometry of poly(ethylene imine)s by electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)

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In this contribution, linear poly(ethylene imine) (PEI) polymers, which are of importance in gene delivery, are investigated in detail by using electrospray ionization-quadrupole-time of flight (ESI-Q-TOF) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS). The analyzed PEIs with different end groups were synthesized using the polymerization of substituted 2-oxazoline via a living cationic ring-opening polymerization (CROP) and a subsequent hydrolysis under acidic conditions. The main goal of this study was to identify linear PEI polymers in a detailed way to gain information about their fragmentation pathways. For this purpose, a detailed characterization of three different linear PEIs was performed by using ESI-Q-TOF and MALDI-TOF MS in combination with collision-induced dissociation (CID) experiments. In ESI-MS as well as MALDI-MS analysis, the obtained spectra of PEIs resulted in fitting mass distributions for the investigated PEIs. In the tandem MS analysis, a 1,2-hydride shift with a charge-remote rearrangement via a four-membered cyclic transition state, as well as charge-induced fragmentation reactions, was proposed as the main fragmentation mechanisms according to the obtained fragmentation products from the protonated parent peaks. In addition, heterolytic and homolytic cleavages were proposed as alternative fragmentation pathways. Moreover, a 1,4-hydrogen elimination was proposed to explain different fragmentation products obtained from the sodiated parent peaks. **Copyright © 2012 John Wiley & Sons, Ltd.**

Supporting information may be found in the online version of this article.

Keywords: tandem mass spectrometry; poly(ethylene imine)s; collision-induced dissociation; fragmentation mechanism; electrospray ionization

INTRODUCTION

Poly(ethylene imine)s (PEIs) represent an important class of cationic polymers that are widely used in different industrial processes such as paper production, shampoo manufacturing, textile industry, water purification, and in the preparation of electrochemical sensors. However, the most current scientifically interesting topic is related to the usage of PEI as a complexation agent for negatively charged DNA and RNA in gene delivery.[1–8] There are several ways to synthesize PEIs, such as the cationic ring-opening polymerization (CROP) of aziridine by a Lewis acid leading to branched PEIs,[9] the controlled polymerization of *N*-substituted aziridines (which avoids branching reactions),[10] and the polymerization of substituted 2-oxazoline via the living CROP and the subsequent hydrolysis under acidic or basic conditions that yields strictly linear PEIs.[11–16] The living CROP of 2-oxazolines (Ox), and the subsequent hydrolyis of the resulting poly(2-oxazoline)s (POx) enables the possibility to synthesize well-defined linear poly(ethylene imine)s with a narrow molar mass distribution. Saegusa *et al.* published the first detailed synthesis and characterization study of linear PEIs starting from the living CROP of Ox and the subsequent hydrolysis of obtained POx under alkaline conditions. Our group recently published several contributions dealing with the synthesis of PEI homopolymers and copolymers for different possible applications such as temperature and pH responsive micelles.[14,15] A large number of studies have been performed on PEI/DNA complexes to investigate the correlation between the transfection efficiency and different characteristics of the PEI polymer, such as purity, molar mass, and architecture (linear and branched). Molar mass has been found to have a significant impact on the transfection efficiency; an increased transfection efficiency is reached with increased molar mass of PEIs.[17–22] However, the toxicity of PEI increases with a prolonged chain length of PEI; hence, the optimal molar mass that provides

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low toxicity but high transfection efficiency has to be elucidated. The *in vivo* application of PEI requires a pure and well-defined material to obtain reproducible transfection efficiency results and to minimize the immunological response of the body to the cationic polymer. Therefore, a controlled synthesis and a detailed characterization of these macromolecules have tremendous importance because of its biological usage.

Mass spectrometry, combined with soft ionization techniques such as electrospray ionization mass spectrometry (ESI-MS)[23] and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS),[24,25] has been widely utilized for the characterization of different polymer samples. Although each method involve different processes in ion formation, both techniques generally allow the ionization of various macromolecules with little or no fragmentation, enabling an accurate molar mass determination by making the unfragmented structure amenable to mass separation. Both techniques are frequently used for the characterization of different polymers for various purposes, such as to determine the accurate molar mass distributions of polymers, to analyze initiating and terminating end groups of different polymers, to study polymerization mechanisms and kinetics, to determine the initiator efficiency in radical polymerizations, to quantify the efficiency of photoinitiation processes in free radical polymerizations, and to identify the degradation products of different polymers.[26–36] The interfacing of these soft ionization methods with collision-induced dissociation (CID) or post-source decay (PSD) methods presents a modern powerful technique for the detailed structural characterization of polymers including the investigation of the sequence and molecular architecture in copolymers.[37–49] Despite the utmost importance of PEI, for transfection applications, only very few detailed characterizations of this material were reported up-to-date. Furthermore, mass spectrometric analyses of PEI are quite rare. This fact could be caused by the multiple charges of this polymer, which render it very suitable for complexation with negatively charged DNA but also introduces additional challenges in the MS characterization. For this study, PEIs were synthesized with different end groups by using the living CROP of 2-ethyl-2-oxazoline (EtOx) and subsequent hydrolysis under acidic conditions. The main goal was to identify these PEI polymers with different instrumentations to gain information about their fragmentation pathways. For this purpose, a detailed characterization of PEIs was performed by ESI-Q-TOF MS/MS and MALDI-TOF MS/MS. Comprehensive information concerning the fragmentation mechanisms of the PEI polymers and comparison of the tandem mass analysis of these polymers with two different ionization techniques (ESI/MALDI) is reported.

EXPERIMENTAL SECTION

Materials

2-Ethyl-2-oxazoline (99%, Acros, EtOx) was dried over barium oxide and distilled under argon prior to use. Methyl tosylate (98%, Aldrich, MeTos) was distilled and stored under argon. Sodium azide (99%, Sigma Aldrich) and acetonitrile (extra dry, Acros) were stored under argon. The matrix material for the MALDI-TOF MS and MS/MS measurements was 2,5-dihydroxybenzoic acid (DHB), and it was purchased from Sigma Aldrich. All solvents used for the ESI-Q-TOF MS measurements were LC-MS grade solvents; they were purchased from Sigma Aldrich and used as received.

Instrumentation

The polymerizations and hydrolyses were performed in a Biotage Initiator Sixty microwave synthesizer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AC 300 MHz at 298 K. Chemical shifts are reported in parts per million (ppm, δ scale) relative to the residual signal of the deuterated solvent. The IR spectra were recorded on a FT-IR spectrometer IRAffinity-1 (Shimadzu).

General procedure for the microwave-assisted polymerization of PEtOx

A stock solution containing the initiator, EtOx monomer, and the solvent (acetonitrile) was prepared. The monomer concentration was adjusted to 4 M; and a monomer to initiator ratio of 20 was used, yielding a molar mass at 2000 g/mol. The microwave vials were heated to 140 °C for a predetermined time in the microwave synthesizer. The polymerization was quenched through the addition of trace amount of water or an excess of sodium azide. Subsequently, the solution was filtrated and the solvent was evaporated in vacuum. The polymer was redissolved in chloroform, washed with water, and precipitated in ice-cold diethyl ether. ^{1}H NMR samples were prepared to determine the monomer conversion and the molar masses (M_n) of the polymers.

Acidic hydrolysis of PEtOx to linear PEIs

Linear PEI polymers were synthesized through acidic hydrolysis of the corresponding PEtOx homopolymers. The PEtOx was heated with an excess of 6 M aqueous HCl in a microwave synthesizer at 130 °C for 1 h. After the removal of the acid under reduced pressure at 130 °C, the residue was dissolved in deionized water followed by the addition of 3 M NaOH until precipitation occurred (pH > 9). The PEI was filtered off and precipitated in deionized water for a second and a third time for an improved purification. After the filtration, the PEI was dissolved in methanol or DMF and precipitated into ice-cold diethyl ether. The white precipitate was filtered off and dried at 50 °C in vacuum. The polymers obtained were characterized by ^{1}H NMR and FT-IR spectroscopy. In every case, the degree of hydrolysis was above 99%, as determined by ^{1}H NMR spectroscopy (further information can be found in the supporting information (SI)). A detailed procedure of the synthesis of PEIs can be found elsewhere.[50]

ESI-Q-TOF MS and MS/MS analysis

PEI samples were analyzed by using a micrOTOF Q-II (Bruker Daltonics) mass spectrometer equipped with an automatic syringe pump, from KD Scientific, for sample injection. The ESI-Q-TOF mass spectrometer was running at 4.5 kV, with a desolvation temperature of 180 °C. The mass spectrometer was operating in the positive ion mode. Nitrogen was used as the nebulizer and drying gas. For the CID experiments, a quadrupole was used for the selection of precursor ions, and argon was used as the collision gas. The collision energy was set according to the MS/MS experiments to be carried out by using the tune collision energy function to identify the best collision energy value for these experiments. The standard electrospray ion (ESI) source was used to generate the ions. The concentration of the samples was 10 μg/mL, and all samples were injected with the use of a constant flow rate (180 μL/h) of sample solution. The solvent was methanol or a methanol/water mixture. There was no salt or acid addition prior to analysis, but ionization occured readily from the sodium content that is naturally present in the

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glass or in the polymer sample. A total of 60 scans/spectrum were averaged, and the quoted *m/z* values are monoisotopic. The ESI-Q-TOF MS instrument was calibrated in the *m/z* range 50–3000 with the use of an external calibration standard (Tunemix solution), which is supplied from Agilent. All data were processed via Bruker Data Analysis software version 4.0.

MALDI-TOF MS and MS/MS analysis

MALDI-TOF MS experiments were performed with an Ultraflex III TOF/TOF (Bruker Daltonics) equipped with a Nd:YAG laser and a collision cell. All spectra were measured in the positive reflector mode. For the MS/MS measurements (LIFT™ mode), argon was used as the collision gas at a pressure of 2×10^{-6} mbar, and the collision energy amounts to 20 keV. The instrument was calibrated prior to each measurement with an external PMMA standard (molar mass 410 or 2500) from PSS Polymer Standards Services GmbH (Mainz, Germany) in the required measurement range. MS and MS/MS data were processed using Flex Analysis 3.0, PolyTools 1.12 (beta version), Data Explorer 4.0, and an isotope pattern calculator. The ion abundances of several scans were summed up to obtain spectra with good signal/noise ratio for TOF MS and MS/MS experiments. The quoted *m/z* values are monoisotopic.

For the sample preparation, 1 μL of PEI solution in methanol (10 mg/mL) and 10 μL of 2,5-dihydroxybenzoic acid (DHB) solution in methanol (10 mg/mL) were mixed (without salt addition), and the dried-droplet sample preparation method was applied.[51]

RESULTS AND DISCUSSION

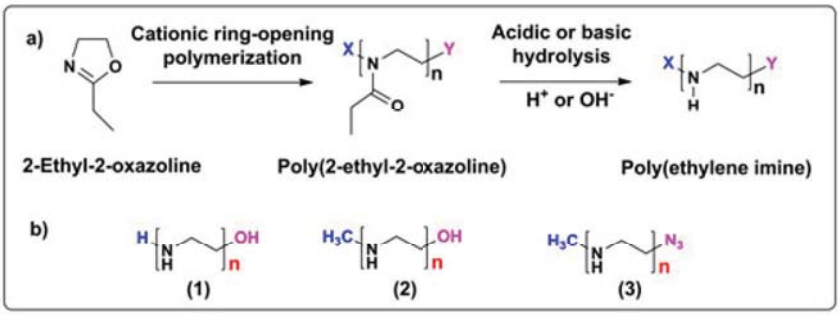
Investigated PEIs

The synthesis of linear PEIs can be performed by hydrolyzing linear PEtOxs under alkaline or acidic conditions. The polymerizations of EtOxs were performed using the microwave-assisted living CROP, providing an easy access to well-defined polymers. As useful end groups a hydrogen starting group, as well as a methyl starting group, and a hydroxyl end group were chosen because of their similarity to those used for transfection agent applications. Furthermore, a methyl starting group and an azide end group were chosen to investigate the behavior and to be able to use PEIs in click reactions.[52,53] A schematic representation of the CROP of EtOx and the subsequent hydrolysis under acidic conditions to obtain PEIs is depicted in Scheme 1(a). The studied PEI polymers are displayed in Scheme 1(b).

The subsequent hydrolysis reaction to obtain the corresponding PEIs was executed in a microwave synthesizer. Previously, the hydrolysis reaction was performed overnight (24 h) under conventional heating to reflux; where, at 99% of the PEtOx, side chains were cleaved off. However, using a microwave synthesizer is much faster, and even azide end groups can tolerate the acidic microwave hydrolysis. After purification, a detailed analysis of the PEI polymers was performed with the use of ^{1}H NMR spectroscopy, showing for all macromolecules a 99% conversion of the amide side chains to amine functionalities (SI and [50]). Further characterization was performed with the use of MS techniques, where ESI-MS and MALDI-MS have been utilized. However, it was a challenging task to characterize the resulting PEIs in MS, as the obtained macromolecules contained large salt contaminations. Complicated spectra rendered the MS analysis almost unfeasible. By applying an optimized purification procedure for the PEI polymers and improved measurement conditions (such as the choice of the matrix for MALDI-MS), we found it possible to obtain analyzable MS, as well as MS/MS spectra. Several MALDI matrices have been used to identify the optimum measurement conditions for PEI polymers, such as *t*-2-(3-(4-*t*-butyl-phenyl)-2-methyl-2-propenylidene)malononitrile (DCTB), caffeinic acid (CA), sinnapinic acid (SA), and 2,5-dihydroxybenzoic acid (DHB). DHB was found to be the best suitable matrix for the characterization of PEI samples, as shortly reported previously in a proceeding publication;[54] therefore, all sample preparations were performed using the DHB matrix.

The compositional information on polymers can be obtained by a single-dimensional mass spectrum that can identify the number and types of monomer units, backbone substituents, and end groups incorporated into the studied polymer. In order to obtain the compositional information, the ESI-Q-TOF and MALDI-TOF mass spectra of the studied PEIs were scrupulously examined in terms of chain end group structures and molar mass distributions.

The PEI polymers, PEI-1, PEI-2, and PEI-3, with different initiating and/or terminating end groups were additionally characterized by utilizing ESI-Q-TOF MS/MS and MALDI-TOF MS/MS to identify the fragmentation products. For this purpose, specific oligomers from the distribution of polymer ions formed upon ionization were mass-selected and fragmented using CID. Tandem mass spectra reveal the masses of the fragmentation products, but not directly their structures or their fragmentation pathways. They can only be elucidated with the knowledge of the



Scheme 1. (a) Schematic representation of the cationic ring-opening polymerization of EtOxs and subsequent hydrolysis into PEIs, (b) schematic representation of the studied PEI polymers 1 to 3.

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corresponding fragmentation mechanisms. All three investigated PEI samples will be discussed in detail in the following section.

Mass spectrometry of PEI-1

ESI-Q-TOF and MALDI-TOF mass spectra of PEI-1 with H and OH end groups at the initiating (*X*) and terminating (*Y*) chain end are depicted in Fig. S1(a)–S1(d) (see SI). Both mass spectra reveal the expected signal spacing correlating to the repeating unit of the investigated PEI (43.04 *m/z* for C_2H_5N monomer unit). The expected PEI chains with hydrogen as initiating end group and hydroxyl as terminating end group were observed as the main distribution in the ESI-Q-TOF and MALDI-TOF mass spectra (labeled as **A** and **B** in Figs. S1(c) and S1(d) (SI)). Peak assignments were achieved on the basis of the expected ion *m/z* values and validated by isotopic pattern information. **A** represents the desired protonated distribution $[H(C_2H_5N)_nOH)+H]^+$, and **B** represents the desired sodiated distribution $[H(C_2H_5N)_nOH)+Na]^+$. The minor distribution (*I*) can be explained by the inefficient hydrolysis of PEtOx to PEI, because these oligomer series still have one EtOx monomer unit in their backbone $[H(C_5H_9NO)_1(C_2H_5N)_nOH)+H]^+$ or $[H(C_5H_9NO)_1(C_2H_5N)_nOH)+Na]^+$ (labeled as **I** in Figs. S1(c) and S1(d) (SI)), which are present in the both ESI-Q-TOF and MALDI-TOF mass spectra to a very little extent. This observation is supported by ^{1}H NMR spectroscopy, revealing a degree of hydrolysis around 99%. The only difference between the ESI-Q-TOF and MALDI-TOF mass spectra arises from some fragments (labeled as Fr in Fig. S1(d) (SI)), which are readily observed in the MALDI-TOF mass spectrum. These fragments were not observed in the ESI-Q-TOF mass spectrum, indicating that these fragments were caused by the MALDI-TOF process.

Tandem mass spectrometry of PEI 1

Figures 1 and 2 represent the ESI-Q-TOF and MALDI-TOF tandem mass spectra of PEI-1. The precursor ion at *m/z* 879.8579 (theoretical *m/z* 879.8618) represents the protonated adduct of the hydrogen-initiated and hydroxyl-terminated oligomer with 20 repeating units $[H(C_2H_5N)_{20}OH+H^+]$. There are several series of product ions, which can be distinguished depending on their newly formed end groups. Various kinds of fragmentation mechanisms can be involved in the formation of these ions. The tandem mass spectra contain four major distributions of fragment ion series ($\mathbf{A}^{\mathbf{X}}_{\mathbf{n}}$, $\mathbf{A}^{\mathbf{Y}}_{\mathbf{n}}$, $\mathbf{B}^{\mathbf{X}}_{\mathbf{n}}$, and $\mathbf{B}^{\mathbf{Y}}_{\mathbf{n}}$) and some minor distributions of fragment ion series ($\mathbf{J}^{\mathbf{VV}}_{\mathbf{n}}$, $\mathbf{J}^{\mathbf{VA}}_{\mathbf{n}}$, and $\mathbf{J}^{\mathbf{AA}}_{\mathbf{n}}$). These fragment ion series may be formed via a charge-remote fragmentation pathway, as shown in Scheme 2. This mechanism involves a 1,2-hydride shift via a charge-remote rearrangement mechanism through a four-membered cyclic transition state, resulting in the formation of two different functionalities at the terminal chain ends. The fragment ions were assigned according to the general nomenclature proposed by Wesdemiotis[55] for synthetic polymers to differentiate fragment ions containing different end groups. Superscripts *X* and *Y* are used to represent ions containing initiating (*X*) or termination (*Y*) end groups, respectively ($\mathbf{A}^{\mathbf{X}}_{\mathbf{n}}$, $\mathbf{A}^{\mathbf{Y}}_{\mathbf{n}}$, $\mathbf{B}^{\mathbf{X}}_{\mathbf{n}}$, and $\mathbf{B}^{\mathbf{Y}}_{\mathbf{n}}$). In this proposed mechanism, CID spectra of protonated PEIs occur through a charge-remote process, in which the ionizing proton does not play an active role in the cleavage. The main fragment series ($\mathbf{A}^{\mathbf{X}}_{\mathbf{n}}$, $\mathbf{A}^{\mathbf{Y}}_{\mathbf{n}}$, $\mathbf{B}^{\mathbf{X}}_{\mathbf{n}}$, and $\mathbf{B}^{\mathbf{Y}}_{\mathbf{n}}$) contain one of the original end groups and new end groups ($-NH_2$ or $-CH=CH_2$), which are formed by the proposed charge-remote fragmentation mechanisms. The *m/z* difference between two sequential fragmentation ions within one series corresponds to 43.04 *m/z* units, representing the mass of one repeating unit of PEI. The most abundant fragment ions series ($\mathbf{A}^{\mathbf{X}}_{\mathbf{n}}$, $\mathbf{A}^{\mathbf{Y}}_{\mathbf{n}}$, $\mathbf{B}^{\mathbf{X}}_{\mathbf{n}}$, and $\mathbf{B}^{\mathbf{Y}}_{\mathbf{n}}$) apparently result from this proposed

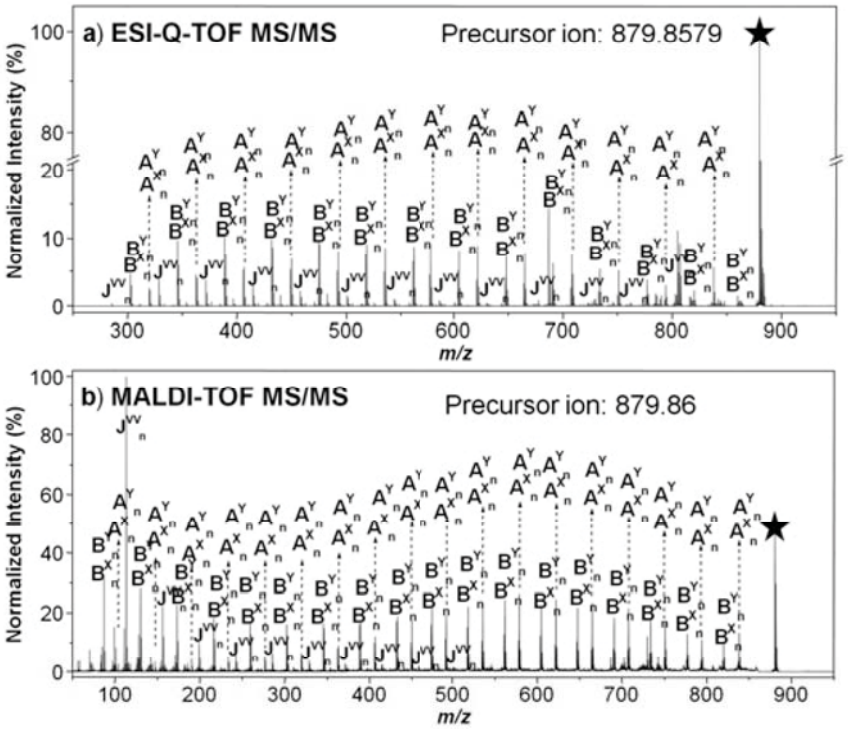

Figure 1. (a, b) ESI-Q-TOF and MALDI-TOF/TOF tandem mass spectra of the protonated PEI-1 parent peak with 20 repeating units marked with a star $[H(C_2H_5N)_{20}OH+H]^+$ (*m/z* 879.8579).

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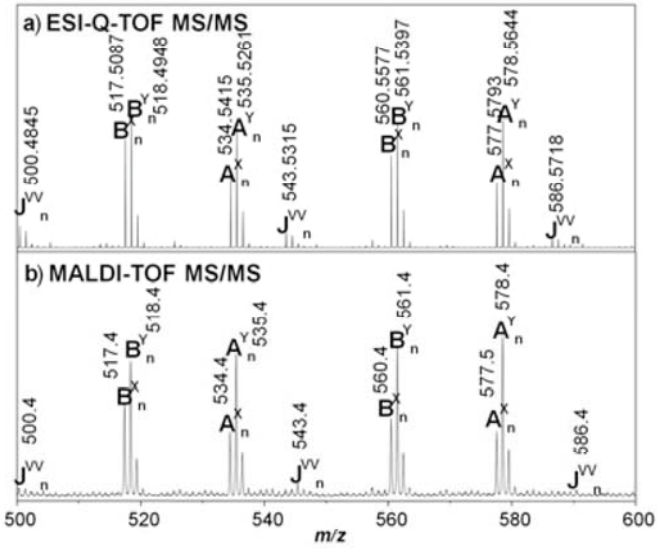
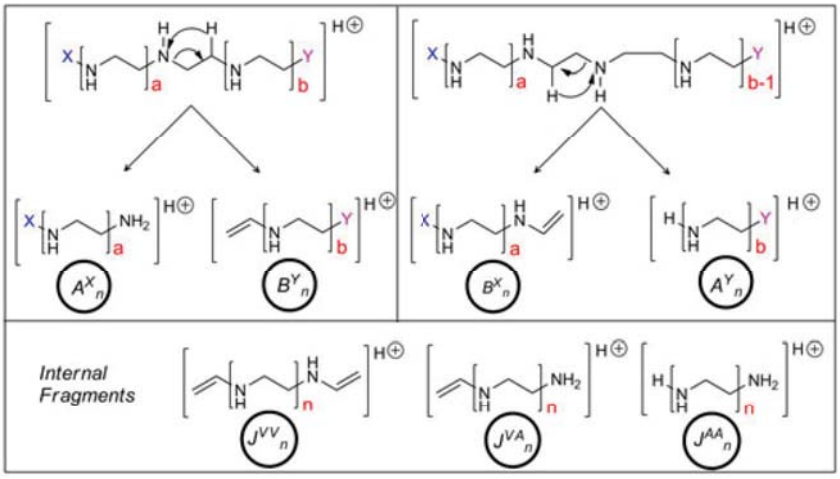


Figure 2. (a, b) ESI-Q-TOF and MALDI-TOF/TOF tandem mass spectra of the protonated PEI-1 and a close up of the range between *m/z* 500 and 600.

mechanism; whereas, the less abundant fragment ion series correspond to internal fragments (J_n^{VV}, J_n^{VA}, and J_n^{AA}) produced by a more complex fragmentation process. This charge-remote fragmentation occurs at both sides of the oligomer chains. Internal fragments with a variety of end groups were named as J_n species with different superscripts that indicate their end groups such as vinyl/amine (J_n^{VA}), vinyl/vinyl (J_n^{VV}), and amine/amine (J_n^{AA}). It must be noted that the ions of the main fragment series can result from both charge-remote and charge-induced dissociation mechanisms, but it is difficult to evaluate the extent of fragment ions from each pathway. Dissociation via competing charge-induced cleavage leads to similar fragments. Charge-induced cleavage of C-N bond followed by an energetically favorable 1,2-hydride shift could be an alternative to the charge-remote fragmentation. Lattimer [56–58] has already described in his earlier studies that a charge-induced dissociation from the protonated PEIs and polyglycols generates some of the fragment ion series via heterolytic or homolytic cleavage (Scheme 3). Polyethylene glycol (PEG) mainly dissociates via a charge-induced fragmentation mechanism and, to a lesser extent, via 1,4-hydrogen eliminations (especially metalated precursor ions). The same situation could be possible for PEI polymers because of the similar backbone. The cationic nature of PEI would imply a charge-induced dissociation at the site where the proton, as charge carrier, is located, as proposed by Lattimer. However, it is noteworthy that the substitution of –O by –NH can significantly change the unimolecular chemistry of the corresponding polymer cation. Because of the larger proton affinity of amines compared to ethers, protonation should take place at the –NH functionality and form an ammonium cation. These ions posses a well-stabilized charge center and might favor the charge-remote fragmentation mechanism upon CID. Moreover, charge-induced fragmentation mechanisms can only explain some of the found fragment ion series. Unfortunately, it is not possible to explain all fragment series with heterolytic or homolytic cleavage mechanisms. The tandem MS analyses of the PEIs were performed under both collision energy conditions (low energy in ESI-CID and high energy in MALDI-CID), yielding fragments, which suggest that the proposed charge-remote fragmentation reactions, as well as the charge-induced fragmentation reactions, are the dissociation mechanisms.

The tandem mass spectra of the protonated and the sodiated PEI precursor ions revealed some differences. A comparison of the ESI-Q-TOF tandem mass spectra of the protonated PEI precursor ions $[M+H]^+$ (observed *m/z* 879.8579, theoretical *m/z* 879.8618) and the sodiated PEI precursor ions $[M+Na]^+$ (observed *m/z* 901.8476, theoretical *m/z* 901.8438) shows that the sodiated precursor ions are more stable than the protonated precursor ions. The protonated species could easily be fragmented at low collision energies (20–60 eV); whereas, the sodiated species could only be fragmented to a small extent even at higher collision energies (60–200 eV). This indicates that sodiated ions are more stable towards collisional dissociation; hence, there is a need to use higher collision energies for obtaining fragments from sodiated species. The fragmentation mechanisms of the collisional dissociation for sodiated and protonated species are also different. The protonated species follow either a 1,2-hydride shift via a charge-remote rearrangement mechanism (Scheme 2) or a charge-induced fragmentation mechanism (heterolytic and homolytic bond cleavage, Scheme 3, similar cleavages were already proposed by Lattimer for PEGs). On the other hand, the sodiated species appear to fragment via a charge-remote 1,4-hydrogen



Scheme 2. Schematic representation of the proposed charge-remote rearrangement mechanisms involved in the fragmentation of protonated PEIs and the representation of potential fragmentation products.

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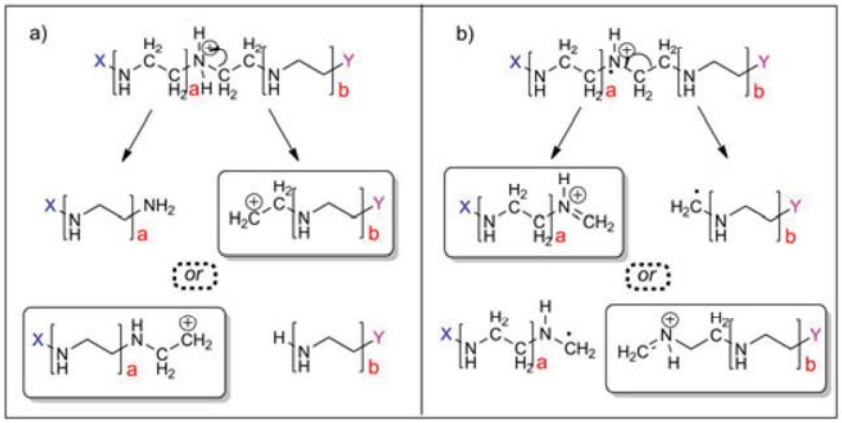
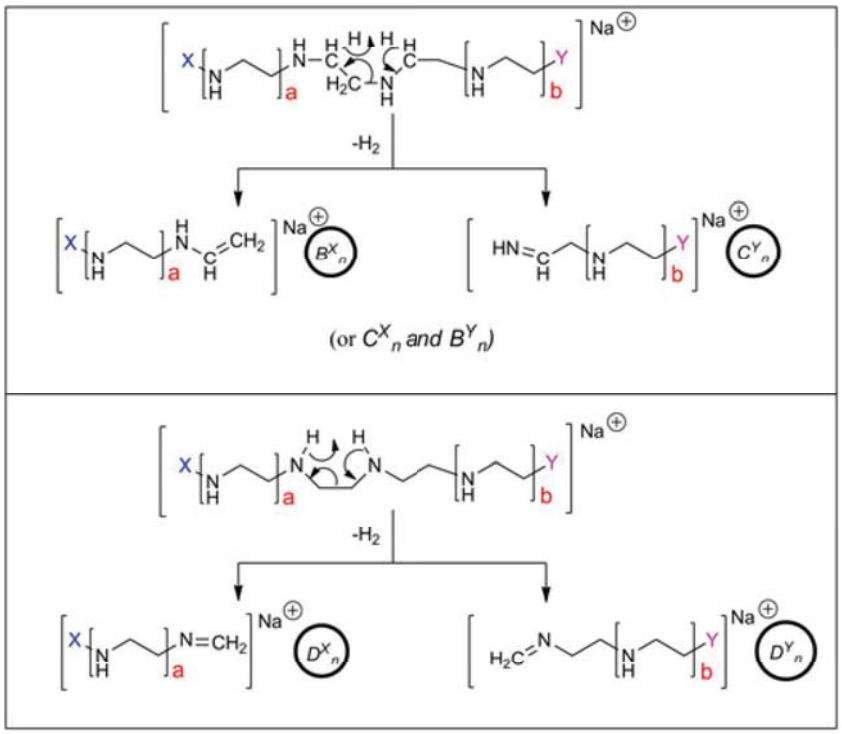


Scheme 3. Schematic representation of (a) heterolytic C-N bond cleavage and (b) homolytic C-C bond cleavage in a PEI chain.

elimination pathway that is analogous to that of PEG (Scheme 4) and a 1,2-hydride shift via charge-remote rearrangement mechanism. For the sodiated parent peaks, possible fragmentation pathways, such as the 1,4-hydrogen elimination, were proposed, and the formed structures (B^X_n, B^Y_n, C^X_n, C^Y_n, D^X_n, and D^Y_n) were correlated to the obtained masses of the fragmentation products. The differences between the sodiated and the protonated species for PEI-1 are depicted in Fig. 3. The schematic representation of the proposed charge-remote 1,4-hydrogen elimination mechanisms involved in the fragmentation of the sodiated PEIs and the resulting fragmentation products are shown in Scheme 4. The fragment product ions from the protonated and sodiated species (Table S1 and Table S2, in SI) are distributed throughout the whole mass range. This observation was valid for both tandem MS measurements (ESI and MALDI MS/MS).

PEI-2 and PEI-3

The ESI-Q-TOF and MALDI-TOF mass spectra of PEI-2, with methyl as initiating end groups and hydroxyl as terminating end groups, and a close up of the ESI-Q-TOF and MALDI-TOF mass spectra of PEI-2 in the range between *m/z* 800 and 900 are depicted in Fig. S2 (SI). **A** represents the protonated distribution of the expected product $[CH_3(C_2H_5N)_nOH)+H]^+$, and **B** represents the sodiated distribution $[CH_3(C_2H_5N)_nOH)+Na]^+$. There are two additional minor distributions obtained in the MS analysis (labeled as **C** and **D** in Figs. S2(c) and S2(d) (SI)). **C** represents the protonated side product distribution $[H(C_2H_5N)_nOH)+H]^+$, and **D** represents the sodiated side product distribution $[H(C_2H_5N)_nOH)+Na]^+$. These side products are formed during the living CROP of EtOx, and they can be explained by known chain transfer reactions during



Scheme 4. Schematic representation of the proposed charge-remote 1,4-hydrogen elimination mechanisms involved in the fragmentation of the sodiated PEIs and the representation of the potential fragmentation products.

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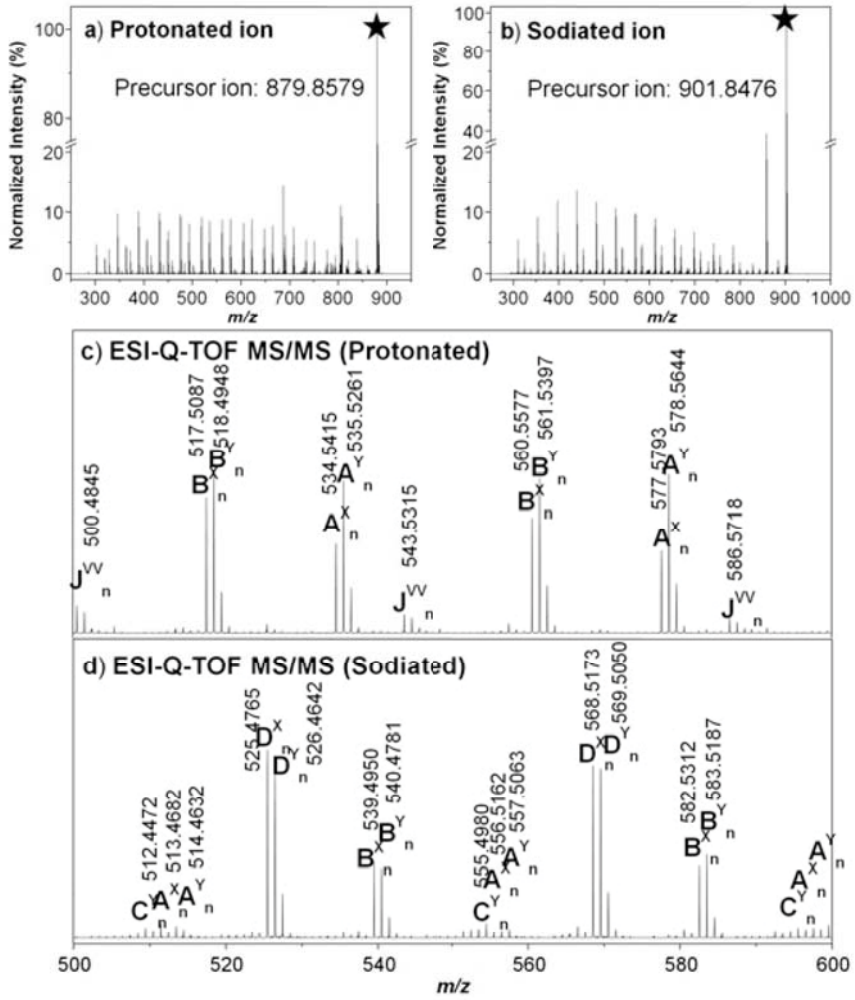

Figure 3. (a, b) ESI-Q-TOF tandem mass spectra of the protonated $[H(C_2H_5N)_{20}OH + H]^+$ (*m/z* 879.8579) and sodiated $[H(C_2H_5N)_{20}OH + Na]^+$ PEI-1 parent peak with 20 repeating units (*m/z* 901.8476) and (c, d) a close up of the ESI-Q-TOF tandem mass spectra of the protonated and sodiated PEI-1 in the range between *m/z* 500 and 600.

the CROP, which lead to proton-initiated PEtOx instead of methyl-initiated PEtOx. These proton-initiated PEtOx polymers are later turned into PEIs side products by acidic hydrolysis. The minor distribution (**I**) can be explained by the inefficient hydrolysis of PEtOx to PEI, as mentioned before. These oligomer series still bear one EtOx monomer unit in their backbone $[CH_3(C_5H_9NO)_1(C_2H_5N)nOH) + H]^+$ or $[CH_3(C_5H_9NO)_1(C_2H_5N)nOH) + Na]^+$ (labeled as I in Figs. S2(c) and S2(d) (SI)). They are present in both ESI-Q-TOF and MALDI-TOF mass spectra to a minor extent. Figure , 4(a)–4(d) represent the ESI-Q-TOF and MALDI-TOF/TOF tandem mass spectra of PEI-2. For the tandem mass analysis, the precursor ion (*m/z* 893.8823), which is the protonated adduct of the methyl-initiated and hydroxyl-terminated oligomer with 20 repeating units $[CH_3(C_2H_5N)_{20}OH + H]^+$, was selected. The fragment ion series can be explained by the previously mentioned charge-remote fragmentation pathway (Scheme 2), which involves a 1,2-hydride shift via a charge-remote rearrangement mechanism through a four-membered cyclic transition state resulting in the formation of two different functionalities ($-NH_2$ or $-CH=CH_2$) at the terminal chain ends.

Figure S3(a)–S3(d) displays the ESI-Q-TOF and MALDI-TOF mass spectra of PEI-3 with methyl as initiating end groups and azide as terminating end groups and a close up of the ESI-Q-TOF and MALDI-TOF mass spectra of PEI-3 in the range between *m/z* 800 and 900 (SI). **A** represents the protonated distribution of the desired product $[CH_3(C_2H_5N)_nN_3) + H]^+$, and **B** represents the sodiated distribution $[CH_3(C_2H_5N)_nN_3) + Na]^+$. Again, there are two minor distributions obtained in the MS analysis, which are formed by chain transfer reaction in the CROP that led to the proton-initiated PEtOx and were later turned into the proton-initiated PEIs during subsequent hydrolysis (labeled as **C** and **D** in Figs. S3(c) and S3(d) (SI)). The comparison of the ESI-Q-TOF and MALDI-TOF mass spectra of PEI-3 shows some differences between these two techniques. In the ESI-Q-TOF mass spectrum, there is one extra distribution (**N**) obtained for this polymer, which is formed because of the decomposition of the azide end group into nitrene through losing nitrogen (shown in Fig. S3(f)).[59] In a number of cases, loss of nitrogen from azides results in uncharged monovalent nitrogen intermediates called nitrenes. Nitrenes have triplet ground states and a short lifetime.[60] There were several additional distributions (**E, F, G**, and **H**) in the MALDI-TOF mass spectrum. A possible explanation for this situation is that the nitrene intermediate is very unstable, and different reactions might occur to stabilize it. The matrix material (2,5-dihydroxybenzoic acid (DHB)) used for this study is acidic. Therefore, it acts as a proton source to promote the

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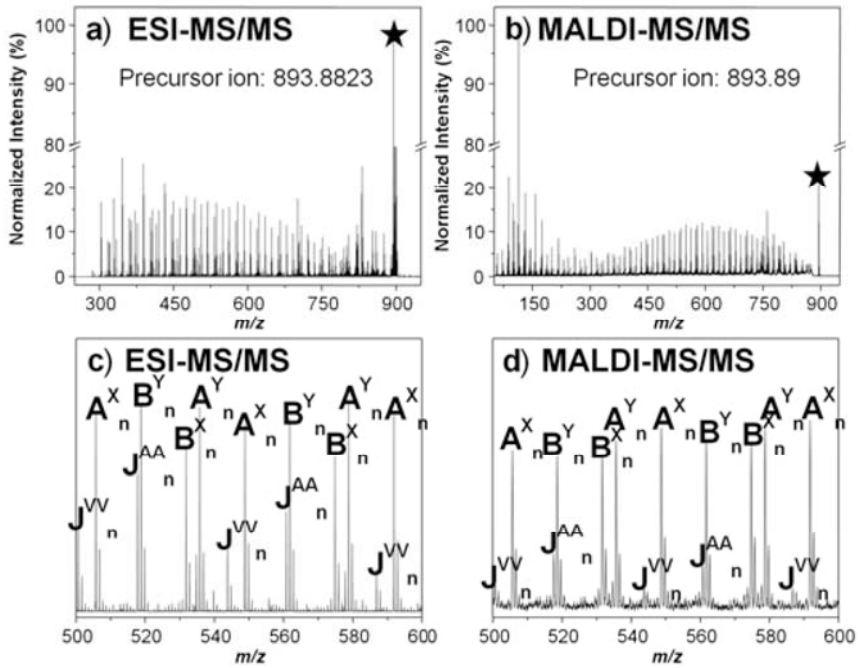

Figure 4. (a, b) ESI-Q-TOF and MALDI-TOF/TOF tandem mass spectra of the protonated PEI parent peak with 20 repeating units marked with a star $[CH_3(C_2H_5N)_{20}OH + H]^+$ (*m/z* 893.8823) and (c, d) a close up of the ESI-Q-TOF and MALDI-TOF/TOF tandem mass spectra of PEI-2 in the range between *m/z* 500 and 600.

formation of amine end groups from these nitrene intermediates. As a consequence, the main distribution in the MALDI-TOF mass spectrum was the PEI with methyl as the initiating end group and amine as the terminating end group. In contrast, the main distribution in the ESI-Q-TOF mass spectrum was the desired PEI structure with methyl and azide end groups, proving that the ESI technique is the softer ionization method, compared to MALDI, for the analysis of fragile azide end groups. The structural explanation of all distributions (**E, F, G**, and **H**) for PEI-3 polymer is depicted in Fig. S3(e) (SI).

The tandem mass experiment was carried out with the precursor ion (observed *m/z* 1069.9859, theoretical *m/z* 1069.9924) that possessed 23 repeating units and with sodium as the ionizing agent $[CH_3(C_2H_5N)_{23}N_3) + Na]^+$. Figure 5(a) and 5(b) represent the ESI-Q-TOF tandem mass spectrum of PEI-3 which showed some differences compared to PEI-1 and PEI-2. In the upper molar mass region of the tandem mass spectrum right after the precursor ion, a pronounced loss of nitrogen was observed. This neutral loss can be assigned to the decomposition of the azide end group into nitrene, which was also observed in the single-dimensional MS analysis.[59] The tandem mass spectrum shows the fragment ion series corresponding to the fragment series formed by a 1,2-hydride shift via charge-remote rearrangement mechanism (Scheme 2) and a charge-remote 1,4-hydrogen elimination pathway (Scheme 4), which are already discussed for the previous PEI polymers, within the medium and low molar mass region. A proper selection of a precursor ion for the fragmentation experiments was not possible because of the multiple overlapping fragments observed in the MALDI-MS; therefore, no tandem MS data were obtained for PEI-3 polymer.

A comparison of the tandem mass spectra of PEIs with different end groups reveals that only the azide end group has a tendency to cleave easily through the loss of nitrogen in the formation of an unstable nitrene end group which can also be turned into an amine end group in the MALDI-MS analysis. On the other hand, hydrogen, hydroxyl, and methyl end groups do not show any direct fragmentation. This behavior can be explained by the leaving group ability of these end groups; they have no tendency to fragment. Therefore, different charge-remote fragmentation mechanisms may occur along the whole

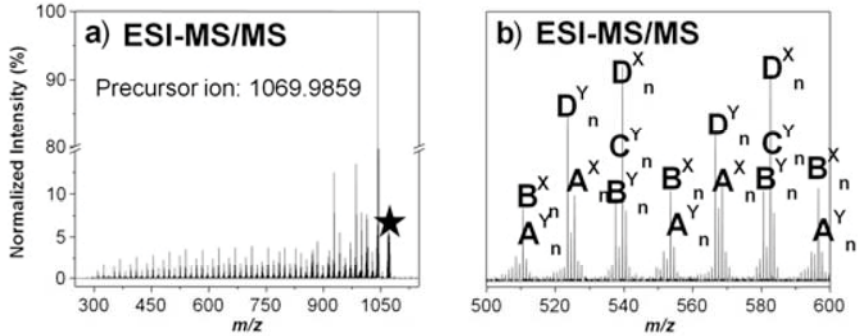

Figure 5. (a) ESI-Q-TOF tandem mass spectrum of the sodiated PEI parent peak with 23 repeating units marked with a star $[CH_3(C_2H_5N)_{23}N_3 + Na]^+$ (*m/z* 1069.9859) and (b) a close up of the ESI-Q-TOF tandem mass spectrum of PEI in the range between *m/z* 500 and 600.

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backbone of the PEIs and form various main fragment series that contain one of the original initiating or terminating end groups (A_n^X, A_n^Y, B_n^X, B_n^Y, C_n^X, C_n^Y, D_n^X, and D_n^Y). Meanwhile, the less abundant fragment ion series can correspond to internal fragments (J_n^{VV}, J_n^{VA}, and J_n^{AA}) produced by a more complex fragmentation process where this charge-remote fragmentation occurs in both sides of the oligomer chains. Overall, it is possible to explain all fragmentation products within the whole molar mass region in the tandem mass spectra of PEIs with these proposed mechanisms and the combination of these mechanisms.

CONCLUSIONS

In this contribution, ESI-Q-TOF and MALDI-TOF mass spectrometers were utilized to elucidate in detail the macromolecular structures of linear PEIs. Tandem mass spectrometry experiments have been performed to investigate the possible fragmentation mechanisms with different initiating and terminating end groups. For PEIs, the type of the cation attached to these polymers was important, because protonated and sodiated species are fragmented via different fragmentation pathways. The presented study provides important information on the fragmentation mechanisms of PEI polymers and the comparison of the tandem mass analysis of these polymers with two different ionization techniques (ESI/MALDI). The information gained from this study will also help to build up a tandem MS product ion library for PEIs with different end groups including fragmentation pathways. This will provide necessary knowledge for the future to make the fast and automated identification of these polymers possible. This kind of studies will form a new research field that can be named as 'Polymeromics', and the obtained information will facilitate the structural elucidation of polymers (i.e. analysis of the initiating/end groups, the accurate molar mass distributions).

Acknowledgements

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Supporting Information

Supporting information may be found in the online version of this article.

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Publication P5:

ESI, APCI, and MALDI tandem mass spectrometry of poly(methylacrylate)s: A comparison study for the structural characterization of polymers synthesized via CRP techniques and the software application to analyze MS/MS data

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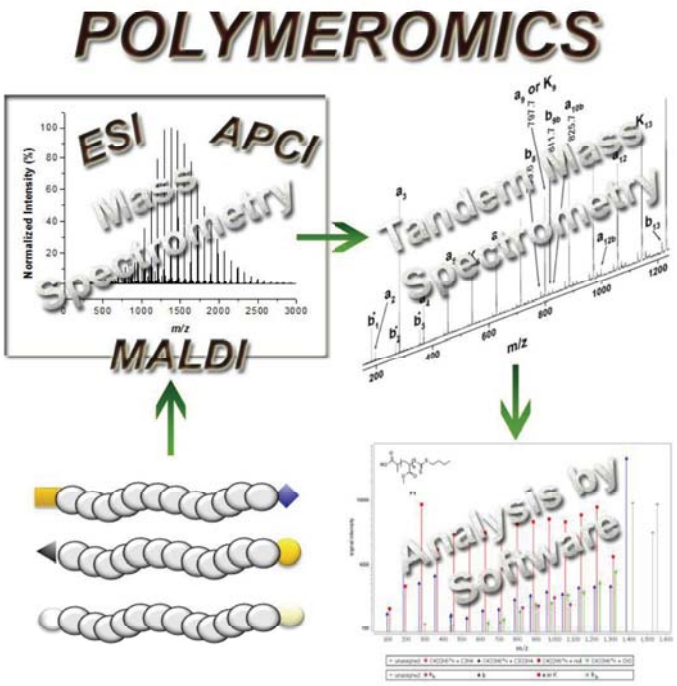
Journal of Polymer Science, Part A: Polymer Chemistry **2013**, *51,* 1595-1605.



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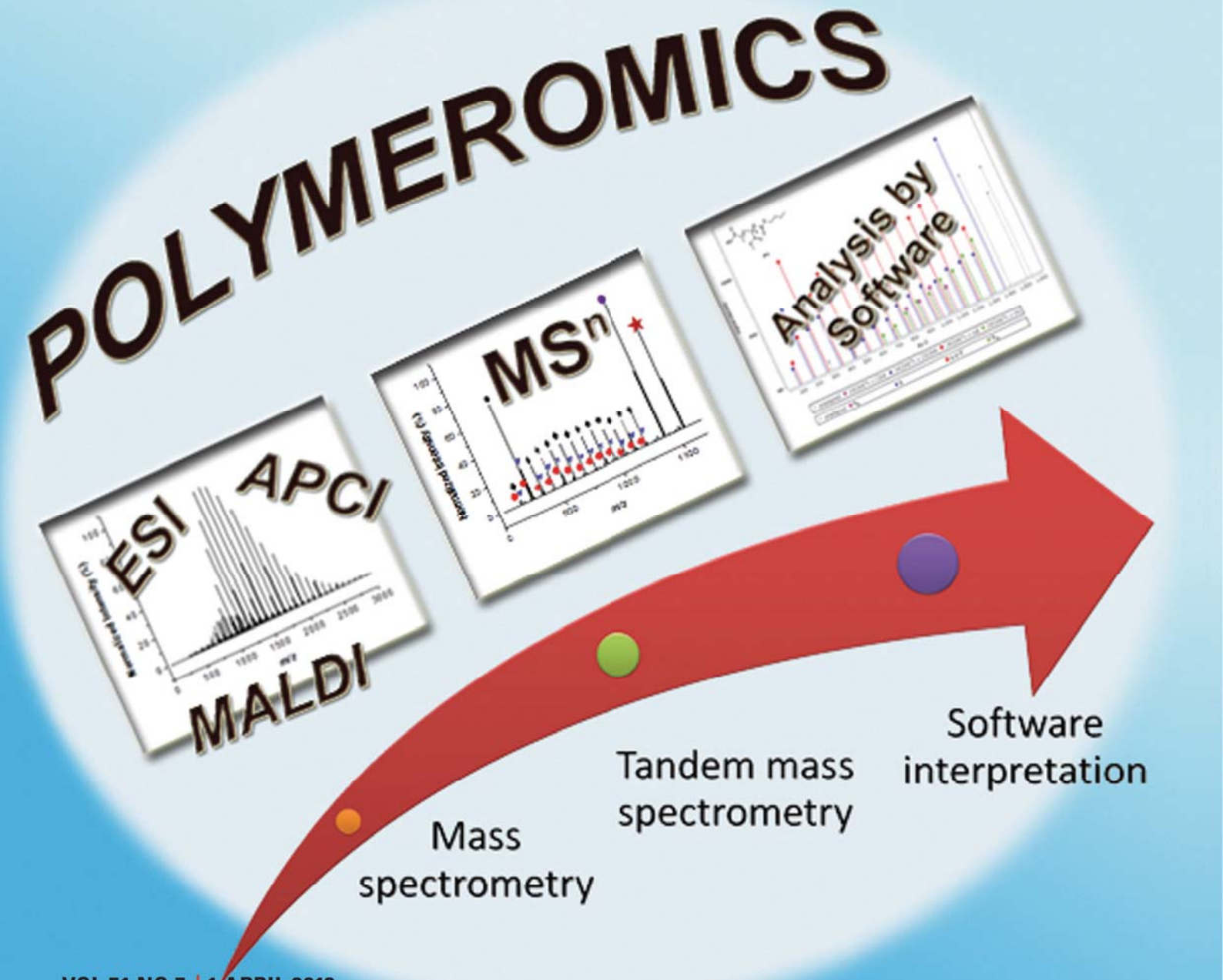

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ESI, APCI, and MALDI Tandem Mass Spectrometry of Poly(methyl acrylate)s: A Comparison Study for the Structural Characterization of Polymers Synthesized via CRP Techniques and the Software Application to Analyze MS/MS Data

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ABSTRACT: Research in polymer science and engineering is moving from classical methodologies to advanced analytical strategies in which mass spectrometry (MS)-based techniques play a crucial role. The molecular complexity of polymers requires new characterization tools and approaches to elucidate the detailed structural information. In this contribution, a comparison study of poly(methyl acrylate)s (PMA) using different tandem mass spectrometry techniques (ESI, APCI, and MALDI MS/MS) is reported to provide insights into the macromolecular structure with the aid of a special MS/MS data interpretation software. Collision-induced dissociation (CID) was utilized to examine the fragmentation pathways of PMAs synthesized via various controlled radical polymerization techniques. All three mass spectrometry techniques are used to analyze structural details of PMAs and the labile end-groups are determined based on the fragmentation behavior in CID. Fragmentation products were identified which are characteristics for the cleavage between the polymer chain and the end-group. The application of a tailor-made software is shown to analyze complex MS/MS data, and it is proven that this kind of software will be helpful for polymer scientists to identify fragmentation products obtained by tandem mass spectrometry similar to the fields of proteomics, metabolomics, genomics, and glycomics. © 2013 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2013**, *51*, 1595–1605

KEYWORDS: APCI; ESI; fragmentation mechanisms; MALDI; poly(methyl acrylate)s; tandem mass spectrometry

INTRODUCTION The characterization of natural and synthetic polymers represents an important part of polymer science, as the main goal is to advance the performance of polymeric material which is associated to its physical properties. Molar mass is one of these properties which affect the resultant characteristics of polymeric material. Determination of molar masses, polydispersity index values, and end-group structures in synthetic polymers is an analytical issue which can be successfully addressed by mass spectrometry (MS). The application of MS to analyze synthetic polymers is a fast and reliable method. A variety of MS techniques have been utilized for the characterization of different macromolecules in recent years because of the development of soft ionization techniques such as electrospray ionization-MS (ESI-MS),[1] matrix-assisted laser desorption ionization-MS (MALDI-MS),[2,3] and atmospheric pressure chemical ionization-MS (APCI-MS).[4] Although these methods involve different processes for ion formation, these techniques generally allow ionization of different macromolecules with little or no fragmentation. Therefore, information about molar mass distributions and end-group functionalities can be obtained. There are several successful examples available in the literature for the combination of some of these methods for the analysis of polymers.[5,6] However, molar mass information alone is not sufficient to derive detailed information of the macromolecular structure of polymers. Additional structural information can be obtained by using tandem mass spectrometry (MS/MS) analysis to identify individual end-groups,

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differentiate isobaric and isomeric species, and to analyze the macromolecular architectures of polymers in detail.[7–10]

Wesdemiotis et al.[11] has recently reviewed the fragmentation pathways of most common synthetic polymer ions. A characteristic fragmentation pattern can be defined for a certain type of synthetic polymer and used as a reference to characterize unidentified polymers within the same class. On the other hand, fragmentation pathways of the same class of synthetic polymers may be different from well-known dissociation behavior in case of polymers with fragile end-groups, for instance, the polymers obtained from different controlled radical polymerization (CRP) techniques. Initiating and/or terminating end-groups can definitely influence chain-end and in-chain bond cleavages of synthetic polymers. As a consequence, it is extremely important to scrutinize the same polymer class with different end-groups to discover the differences in fragmentation pathways. In the present contribution, different mass spectrometry (ESI-Q-TOF, APCI-Q-TOF, and MALDI-TOF MS) instruments were employed to elucidate the fragmentation pathways of different poly(methyl acrylate)s (PMA) which are synthesized via CRP techniques such as atom transfer radical polymerization (ATRP),[12–15] nitroxide-mediated polymerization (NMP),[16,17] and the reversible addition-fragmentation chain transfer (RAFT) process.[18,19] All chain end-groups obtained in each CRP techniques were detected by all three mass spectrometry techniques. MS/MS experiments were performed upon precursor ions of interest to gain further structural information from the resulting fragmentation patterns and to understand the fragmentation mechanism of these PMAs. The information gained from this study will also help to construct a MS/MS product ion library of poly(acrylate)s with different end-groups including fragmentation pathways which will provide the necessary knowledge to allow, in future, a fast and automated identification of these polymers possible with the help of dedicated software solutions. This software will help to characterize synthetic polymers by MS/MS and, thereby, allowing its frequent application as in the fields of proteomics, metabolomics, genomics, and glycomics. This kind of studies will be part of the recently introduced research field "Polymeromics"[10] and the obtained information will facilitate the structural elucidation of various synthetic polymer classes.

EXPERIMENTAL

Materials

Methyl acrylate (MA, Aldrich, 99%) was passed through a column filled with inhibitor remover (Aldrich) to remove the stabilizing hydroquinone prior to polymerization. 2,2′-Azobis(2-methylpropionitrile) (AIBN, Aldrich) was recrystallized from methanol. 2-Cyano-2-butyldithiobenzoate (CBDB) was synthesized according to the previously published literature.[20] The matrix materials *trans*-3-indoleacrylic acid (IAA, Sigma Aldrich), *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB, Sigma Aldrich), 2-(4-hydroxy-phenylazo)benzoic acid (HABA, Sigma Aldrich), 2,5-dihydroxybenzoic acid (DHB, Sigma Aldrich), α-cyano-4-hydroxycinnamic acid (HCCA, Sigma Aldrich), and retinoic acid (RA, Sigma Aldrich), as well as sodium iodide (Sigma Aldrich) and the solvents, chloroform, tetrahydrofuran (THF), and acetone (HPLC grade, Roth) were used as purchased. All solvents utilized for the ESI-Q-TOF MS measurements were LC-MS-grade solvents, purchased from Sigma Aldrich, and used as received.

Size-Exclusion Chromatography Measurements

Size-exclusion chromatography (SEC) was performed on a Shimadzu system equipped with a SCL-10A system controller, a LC-10AD pump, a RID-10A refractive index detector, a SPD-10A UV detector at 290 nm, and a PSS SDV column with chloroform-triethylamine-2-propanol (94:4:2) as eluent and the column oven was set to 50 °C. Poly(methyl methacrylate) (PMMA) samples were used as calibration standards.

Synthesis of PMA via RAFT Polymerization, [MA]/[CTA] = 50/1

A 2 M solution of 0.5 mL MA (239 mg, 5.55 mmol) in anisole with 26 mg CBDB (0.11 mmol) and 3.6 mg AIBN (0.022 mmol) was flushed with argon for 30 min in a capped microwave vial. The mixture was placed in an oil bath heated to 70 °C for 6 h. The polymer was obtained after precipitation into methanol (MeOH) and dried under vacuum until mass constancy was reached.

Synthesis of PMA via NMP Technique, [MA]/[MAMA-SG1] = 50/1

A 4 M solution of 1 mL MA (955 mg, 11.1 mmol) in anisole with 84.7 mg *N*-(2-methylpropyl)-*N*-(1-diethylphosphono-2,2-dimethylpropyl)-*O*-(2-carboxylprop-2-yl)hydroxylamine (MAMA-SG 1) (0.222 mmol) was flushed with argon for 30 min in a capped microwave vial. The mixture was placed in an oil bath heated to 120 °C for 5 h. The polymer was obtained after precipitation into MeOH and dried under vacuum until mass constancy was reached.

Synthesis of PMA-Br via ATRP Technique, [MA]/[I] = 50/1

A 4 M solution of 1 mL MA (955 mg, 11.1 mmol) in anisole with 16 mg copper(I)bromide (CuBr) (0.222 mmol), 17 μL *N,N,N′,N′,N*-pentamethyldiethylenetriamine (PMDETA) (13 mg, 0.11 mmol) and 33 μL ethyl 2-bromoisobutyrate (43 mg, 0.22 mmol) was flushed with argon for 30 min in a capped microwave vial. The mixture was placed in an oil bath heated to 70 °C for 3 h. The polymer was obtained after precipitation into MeOH and dried under vacuum until mass constancy was reached.

Synthesis of PMA-Cl via ATRP Technique, [MA]/[I] = 50/1

A 6 M solution of 1.135 mL MA (1.08 g, 12 mmol) in anisole with 30 mg copper(I)chloride (CuCl) (0.3 mmol), 6 mg copper(II)chloride ($CuCl_2$) (0.044 mmol), 84 μL PMDETA (64 mg, 0.56 mmol), and 28.6 μL methyl 2-chloropropionate (MeCliP) (31 mg, 0.25 mmol) was flushed with argon for 30 min in a capped microwave vial. The mixture was placed in an oil bath heated to 60 °C for 2 h. The polymer was obtained after precipitation into MeOH and dried under vacuum until mass constancy was reached.

ESI-Q-TOF-MS and MS/MS Measurements

PMA samples were analyzed by using a micrOTOF Q-II (Bruker Daltonics) mass spectrometer equipped with an automatic syringe pump from KD Scientific for sample injection. The ESI-Q-TOF mass spectrometer was running at 4.5 kV, at a desolvation temperature of 180 °C. The mass spectrometer was operating in the positive ion mode. Nitrogen was used as the nebulizer and drying gas. For the CID experiments, a quadrupole was used for the selection of the precursor ions and argon was used as the collision gas. The collision energy was set according to the MS/MS experiments to be carried out by using the tune collision energy function to identify the best collision energy value for these experiments. The standard electrospray ion (ESI) source was used to generate the ions. The concentration of the samples was 10 μg/mL and all samples were injected using a constant flow rate (3 μL/min) of sample solution. The solvent was a chloroform/acetonitrile mixture. There was no salt or acid addition prior to analysis, but ionization occurs readily from the sodium content that is naturally present in the glass or in the polymer sample. A total of 60 scans/spectrum were averaged and the quoted *m/z* values are monoisotopic. The ESI-Q-TOF-MS instrument was calibrated in the *m/z* range 50–3000 using a calibration standard (Tunemix solution) which is supplied from Agilent. All data were processed via Bruker Data Analysis software version 4.0.

APCI-Q-TOF-MS and MS/MS Measurements

APCI-Q-TOF-MS measurements were performed with a micrOTOF Q-II (Bruker Daltonics) mass spectrometer equipped with an APCI source. APCI-MS was operated with the corona discharge current set to 4000 nA and the capillary voltage was set to 4000 V. Nitrogen was used as nebulizer and drying (desolvation) gas. The vaporizer temperature was set to 400 °C, the drying (desolvation) gas to 250 °C and 4 L/min, and the nebulizer pressure was 2.0 bar. An Agilent 1200 Series LC system was utilized to dilute the polymer solutions coming from the syringe pump with the help of a T-fitting. The T-fitting was used to mix the polymer solution (0.5 mg/mL) from the syringe pump (360 μL/h) and the acetonitrile/water solvent mixture (50/50, v/v %, 0.4 mL/min) from the LC system before the MS interface. Tandem mass (MS/MS) experiments were conducted by isolating the precursor ion of interest in the quadrupole and subjecting it to CID.

MALDI-TOF MS and MS/MS Measurements

MALDI-TOF MS measurements were performed with an Ultraflex III TOF/TOF (Bruker Daltonics) equipped with a Nd:YAG laser and a collision cell. All spectra were measured in the positive reflector mode. For the MS/MS mode, argon was used as collision gas at a pressure of 2×10^{-6} mbar. The instrument was calibrated prior to each measurement with an external PMMA standard $H(CH_2CCH_3COOCH_3)nH$ + Na+ (m/z = 425 or 2526, measured with sodium iodide) from PSS Polymer Standards Services GmbH in the required measurement range. MS and MS/MS data were processed using the software Flex Analysis and an isotope pattern calculator from Bruker Daltonics and PLUMS, the previously developed tandem mass data interpretation software (http://bio.informatik.uni-jena.de/software/plums).[21]

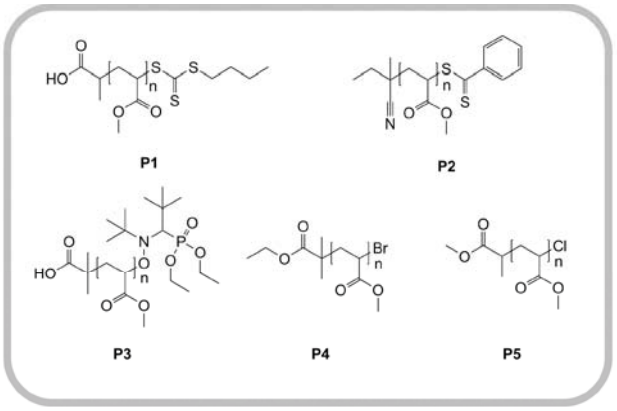
For the sample preparation, the different PMAs in chloroform (10 mg/mL), the matrices (IAA, DCTB, DHB, HABA, HCCA, and RA) in chloroform or THF (each 20 or 30 mg/mL), and the doping salt (NaI) dissolved in acetone at a concentration of 100 mg/mL were used. The dried-droplet spotting technique (matrix, analyte, and sometimes salt previously mixed together) was applied. For each sample, 1 μL of the mixture was spotted onto a target plate.

RESULTS AND DISCUSSION

The polymerization conditions employed for the synthesis of investigated PMA polymers are described in **EXPERIMENTAL** section and their structures are shown in Scheme 1 (**P1** and **P2** via RAFT, **P3** via NMP, and **P4** and **P5** via ATRP).

Advanced MS techniques, such as ESI-Q-TOF MS, APCI-Q-TOF MS, and MALDI-TOF MS, were applied for the analysis of the synthesized PMAs to confirm the incorporation of the end-groups of these polymers. MALDI-TOF MS measurements were performed using six different matrix substances (IAA, DCTB, DHB, HABA, HCCA, and RA) to obtain the best ionization efficiency for PMA. In the case of DCTB as matrix, a salt additive had to be used additionally to achieve ionization of the investigated PMA homopolymers. An overview of the MALDI-TOF MS results with different matrices is exemplarily shown in Figure 1(a–f) for homopolymer **P1**.

For all measurements, the lowest possible laser intensity, which resulted in a good spectrum, was chosen as final laser intensity. Comparing the mass spectra, it is clearly visible that IAA as well as HCCA delivered the best results for this polymer type. The applications of IAA and HCCA matrices cause less in-source fragmentation and provide better signal-to-noise (S/N) ratios. The main distribution belongs, in most cases, to the desired polymer structure and a sodium cation, which is left over from the polymerization. The smaller signal series can be explained by already fragmented polymer chains of the series $\mathbf{a_n}$, which corresponds to closed-shell fragments containing the initiating (α) end-group produced



SCHEME 1 Schematic representation of the structures of the investigated PMA homopolymers **P1–P5**.

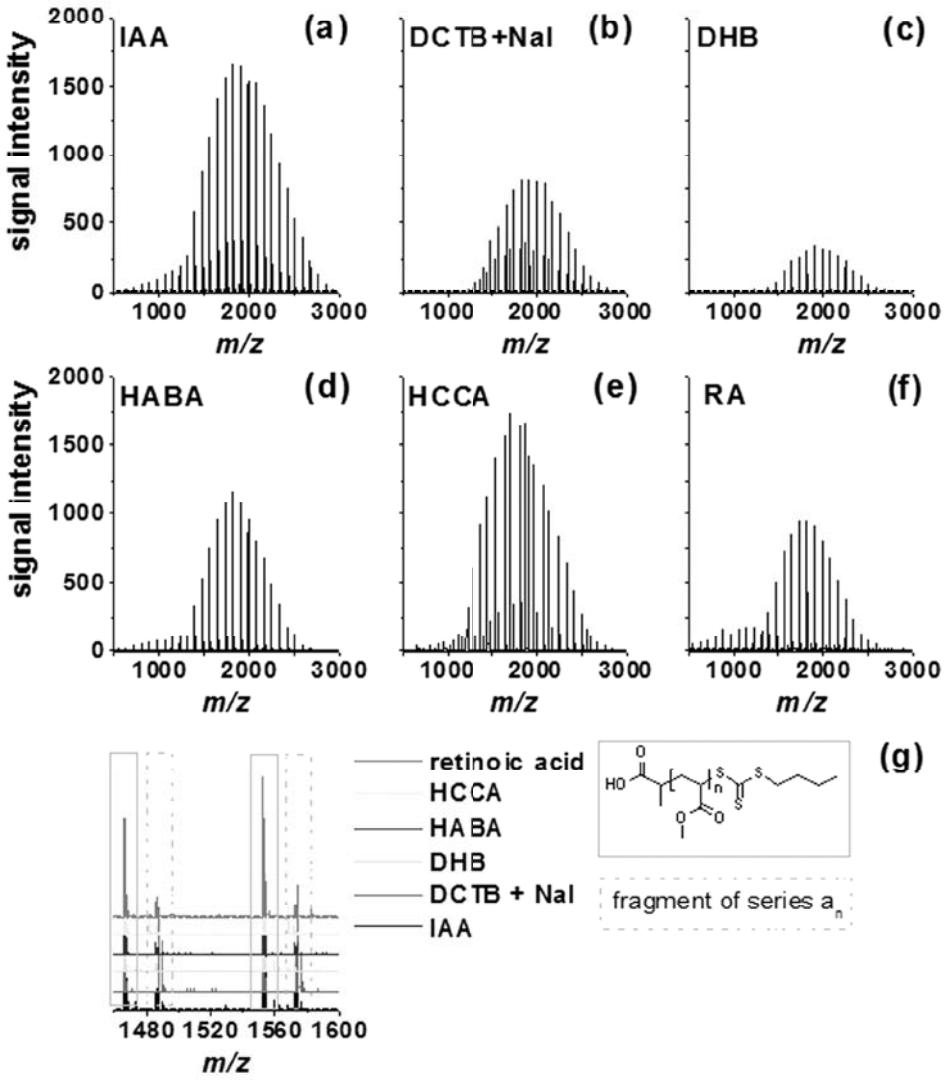


FIGURE 1 MALDI-TOF MS spectra of **P1** using different matrices: (a) IAA; (b) DCTB + NaI; (c) DHB; (d) HABA; (e) HCCA; (f) RA; (g) zoom of the MALDI-TOF MS spectra between *m/z* 1460 and 1600.

by consecutive dissociation of the primary radical ion series.[22] This series might be observed owing to the side products of the polymerization (vinyl-terminated PMAs); however, they were almost invisible in ESI and APCI analysis which confirms the fragmentation of the labile end-group during the MALDI process. This series was observed in the MALDI analysis; on the other hand, they were not observed in the ESI and APCI spectra. Combining these results suggests that this series is an instrumental artifact caused by fragmentation of end-groups during the MALDI process and is not a side product from the polymerization reaction. Furthermore, it is worth to mention that the application of DCTB together with NaI as salt additive provided only low signal intensities corresponding to the desired homopolymer structure, which is shown in Figure 1(g). The main distribution for this kind of matrix belongs to the fragment species $\mathbf{a_n}$, and these findings clarify the importance of the matrix choice. Therefore, IAA is chosen as matrix substance for further MALDI-TOF MS measurements of the other PMA samples (without salt). Additionally, it should be noted that in comparison to the previous studies in this field, the fragile end-groups, such as —Br, —Cl or the RAFT end-groups, are still detectable, which argues for an intact terminating (ω) end-group at the polymer chains under high energy conditions.[6,23] We showed in this study that it is possible to minimize the fragmentation of a labile end-group by optimizing the sample preparation and the measurement conditions in the MALDI-MS analysis.

Typical mass spectra obtained using ESI-Q-TOF MS, APCI-Q-TOF MS, and MALDI-TOF MS for **P1** are shown in Figure 2. The main distribution in all MS analysis can be assigned to the desired polymer structure having both initiating (α) and terminating (ω) end-groups, whereas being ionized with a sodium cation (in ESI and MALDI analysis) and a NH_4^+ ion (or H_2O^+ ion) (in the APCI analysis). Thus, synthesized PMA polymers have a well-defined structure without any

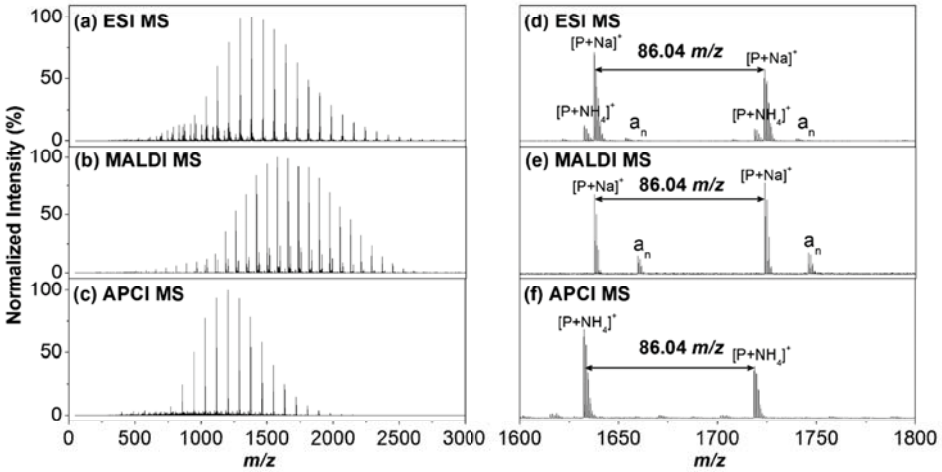


FIGURE 2 (a, b, c) ESI-Q-TOF, MALDI-TOF, and APCI-Q-TOF mass spectra of **P1**, (d, e, f) zoom into the ESI-Q-TOF mass spectra of **P1** in the range between *m/z* 1600 and 1800.

detectable structural defects. The existence of possible structural imperfections would create a deviation of *m/z* values and different isotope pattern distributions, which was not the case in this study. Figure 2(d–f) shows the MS spectra between 1600 and 1800 *m/z* of a typical selection of the molar mass distribution from the polymer sample **P1**. The desired polymer distribution peaks are separated by an interval corresponding to a MA repeating unit (86.04 *m/z*). The number of chain end-groups detected is higher by ESI and APCI than by MALDI MS in most of the cases. Although multiple charged species are often observed in ESI-MS in the literature, there was no such effect observed in our experiments (only doubly charged species were observed in a very little extent in the lower mass region without creating any problems in the interpretation of the signals). The ion distributions in MALDI-TOF and ESI-Q-TOF measurements are more representative of the actual molar mass distributions. However, because of the tendency of APCI-Q-TOF mass spectrometry to favor the ionization of lower molar mass species,[24] the APCI MS spectra should not be considered to represent the true molar mass distribution (M_n^{Theor}: 1786 g/mol, M_n^{SEC}:1850 g/mol, M_n^{MALDI}: 1950 g/mol, M_n^{ESI}: 1690 g/mol, and M_n^{APCI}: 1260 g/mol). Molar masses and PDI values of the all investigated PMAs via different methods (SEC, ESI, APCI, and MALDI) are shown in Supporting Information Figure S1 and Supporting Information Table S1. APCI-MS represents an advantageous technique to analyze fragile end-groups owing to its ability to ionize intact structures; on the other hand, it is inadequate to obtain molar mass information from larger macromolecules owing to the mass discrimination effect which causes the underestimation of average molar masses. Therefore, APCI is not suited for molar mass determination for the samples with molar masses higher than 1.500 g/mol. ESI-MS can be used for molar mass determination up to certain values (with new instrumentations up to 10,000–20,000 g/mol); however, for high molar mass polymers (>5000 g/mol) multiply charged species might cause problems and the deconvolution of the mass spectra must be obligatory to obtain accurate molar mass values. MALDI has the potential to allow the determination of molar mass distributions for synthetic polymers exceeding 30,000 g/mol. However, the observation of molecular species without fragmentation, and resolution of individual oligomers, can be demonstrated successfully only for low molar mass polymers. Moreover, the reliable average molar masses can only be determined directly from the MALDI mass spectra of polymers with relatively narrow molar mass distributions (PDI < 1.3).

The macromolecular structure was confirmed by comparing the obtained results with the theoretical isotope pattern distributions. The presence of other ion series was observed in the ESI mass spectra and this can be attributed to the different cationizations (e.g., Na^+, NH_4^+ and K^+). The observed species were concisely named with the formula of $[P+(Cat)n]^{n+}$, where "P" symbolizes the polymer molecule, "Cat" stands for a cationization agent (which can be sodium, potassium, lithium, proton, ammonium, or water cluster ions), and "*n*" for the charge state of the molecule. The series of less intensive peaks in the MALDI-MS analysis can be ascribed to the species which might form owing to the occurrence of the end-group fragmentation during the MALDI process. ESI and APCI methods were found to be comparable in respect of end-group determination and both techniques generally resulted in less in-source fragmentation compared to MALDI technique. Therefore, ESI and APCI should be considered as method of choice to identify the maximum numbers of end-group functionalities within given polymer samples. In this study, the investigated polymer samples apparently retain perfect or near perfect chain-end functionalities. However, in the case of side products and certain extent of chain-end loss products, it is important to utilize the softest ionization technique to detect all possible polymerization products. ESI and APCI techniques were found to be more efficient in detecting distinct main and side products present in the polymer samples compared to

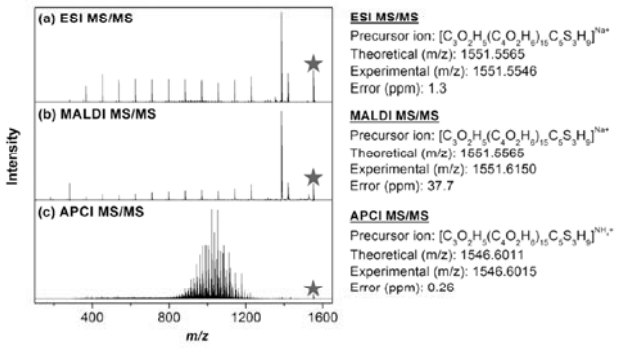


FIGURE 3 (a) ESI-Q-TOF, (b) MALDI TOF/TOF, and (c) APCI-Q-TOF tandem mass spectra of the **P1** parent peak with 15 repeating units.

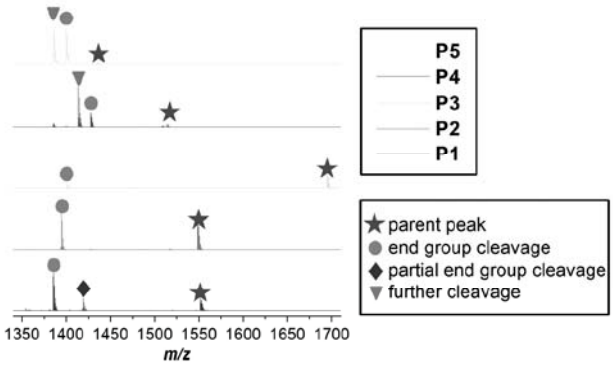


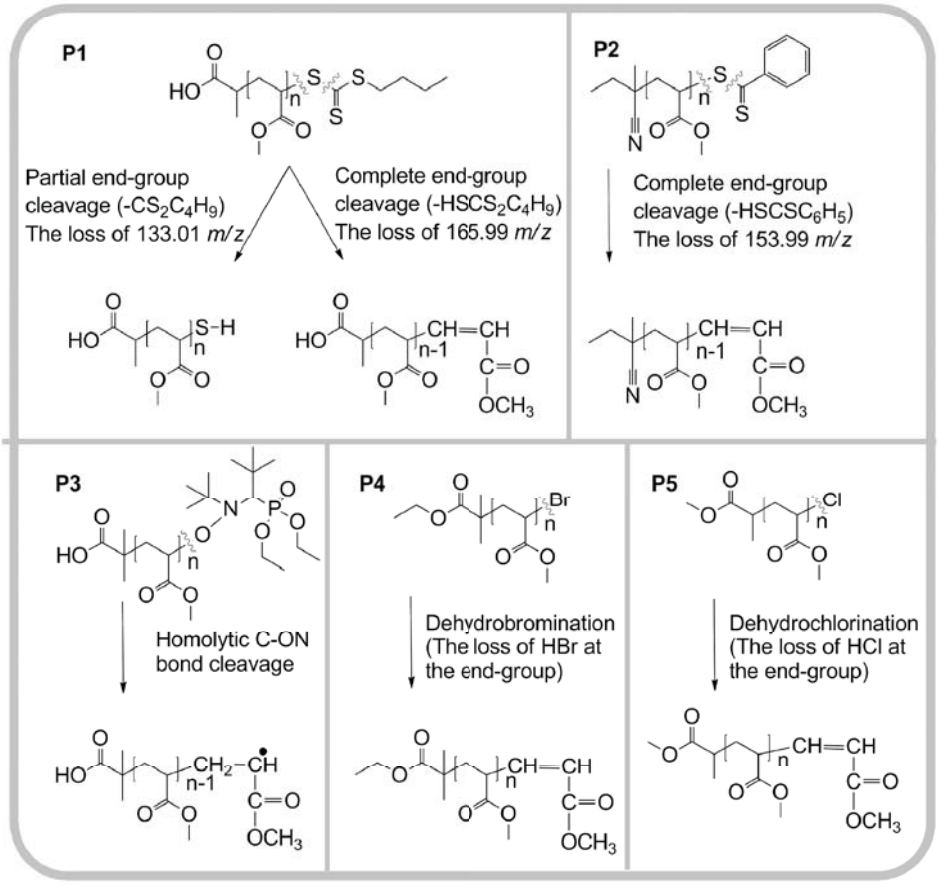
FIGURE 4 Comparison of the fragmentation behavior owing to the different starting and end-groups of all investigated homopolymers: ESI-Q-TOF tandem mass spectra of the **P1** (upper mass region).

the MALDI technique. However, to obtain molar mass information from larger macromolecules to gain complete picture of the polymerization, ESI, and APCI techniques should be used as complementary techniques to MALDI.

The assignment of the observed peaks was further confirmed by the application of tandem MS measurements, because mass data alone are not sufficient to analyze compositional or structural insights of unknown polymers or the polymers synthesized via new synthetic techniques. Therefore, the polymer ions must be isolated and activated (with collision energy) to create diagnostic fragmentation products. The interpretation of the resulting fragmentation product ions helps to reconstruct the primary structure of the selected polymer. In this comparison study, ESI and MALDI MS/MS provided similar results, but the APCI MS/MS data revealed a different and unusual fragmentation mechanism (the reason for this behavior might be the different cationization agent in the APCI analysis). To evaluate the effect of the molar mass on the fragmentation mechanism, different parent peaks (parent peaks: 5RU, 10RU, 15RU, 20RU, 25RU, and 30RU) were compared and they show the same fragmentation mechanisms (data not shown). Sodiated PMA species always generate the same product ions regardless of the size of the precursor ion. For a better assessment of the MS/MS spectra obtained by the different MS techniques, the *m/z* signal of 15 MA repeating units with the corresponding end-groups was chosen as parent peak ion for the investigated polymer to demonstrate the differences between different MS/MS techniques (Fig. 3). ESI and MALDI MS/MS result in similar fragmentation products, whereas APCI MS/MS provide very complicated tandem mass spectra without backbone cleavages. Fragmentation mechanisms observed during CID of the PMA polymers provide straightforward structural information. The primary neutral losses from the precursor ion indicate the end-group masses and the number of 86.04 *m/z* neutral losses provide information about the repeating unit structure. However, in the APCI MS/MS analysis, only small-molecule eliminations (such as water and/or ammonium and methanol) were detected, and thus fragmentation product ion series with an interval corresponding to a MA repeating unit (86.04 *m/z*) were not obtained in this case. It was not completely possible to understand the fragmentation behavior in the APCI-MS/MS analysis from the complicated tandem mass data. Therefore, it is required to perform further APCI-MS experiments with ion-mobility MS to separate possible isomers and isobars to identify the correct cationization agent on polymer ions and APCI-MS/MS experiments to identify fragmentation product ions.

ESI and MALDI tandem mass spectra of parent ions reveal a highly intense fragment ion which is caused by the fragmentation of labile ω end-groups. To illustrate the cleavages of the ω end-groups under CID conditions, the high molar mass region next to the parent peak of the tandem MS spectra is shown for all investigated polymers in Figure 4 and the proposed end-group cleavages are shown in Scheme 2.

In Figure 5, an overview of the fragmentation behavior of the **P1** measured by ESI-Q-TOF MS/MS is shown. At a first glance, five main fragment series could be observed in the low molar mass region of the MS/MS spectra independent of the polymerization technique. The nomenclature of the MS/MS fragments is adopted from other MS/MS homopolymer studies[22,25] and the recent review from Wesdemiotis et al.[11] Nevertheless, the fragmentation mechanism is not exactly the same in these studies owing to the fragile end-groups; it is still possible to explain fragmentation products with the same nomenclature. The letter **a** or **b** indicates these fragments retain the initiating chain-end and the subscripted ***n*** value provides the number of repeating units contained in the fragment. Internal fragmentation product ions (both original end-groups are missing) arising from further dissociations were named with the middle letter from alphabet such as **K** and **J**. Besides the series $\mathbf{a_n}$ and $\mathbf{b_n}$ (from depolymerization), fragment series $\mathbf{a_{nb}}$, $\mathbf{b_{nb}}$, $\mathbf{K_n}$, and $\mathbf{J_n}$ could be identified. A possibility for the formation of the fragment series $\mathbf{b_{nb}}$ is starting from the secondary radical ions $\mathbf{b_n}\bullet$, followed by a β C—C bond scission to release the polymer side group and a hydrogen movement to the polymer chain-end to form the closed-shell fragment (Scheme 3). This mechanism is similar to the already explained fragmentation pathway for the series $\mathbf{a_{nb}}$. However, the dissociation of the side groups



SCHEME 2 Schematic representation of the proposed end-group cleavages for the investigated PMAs.

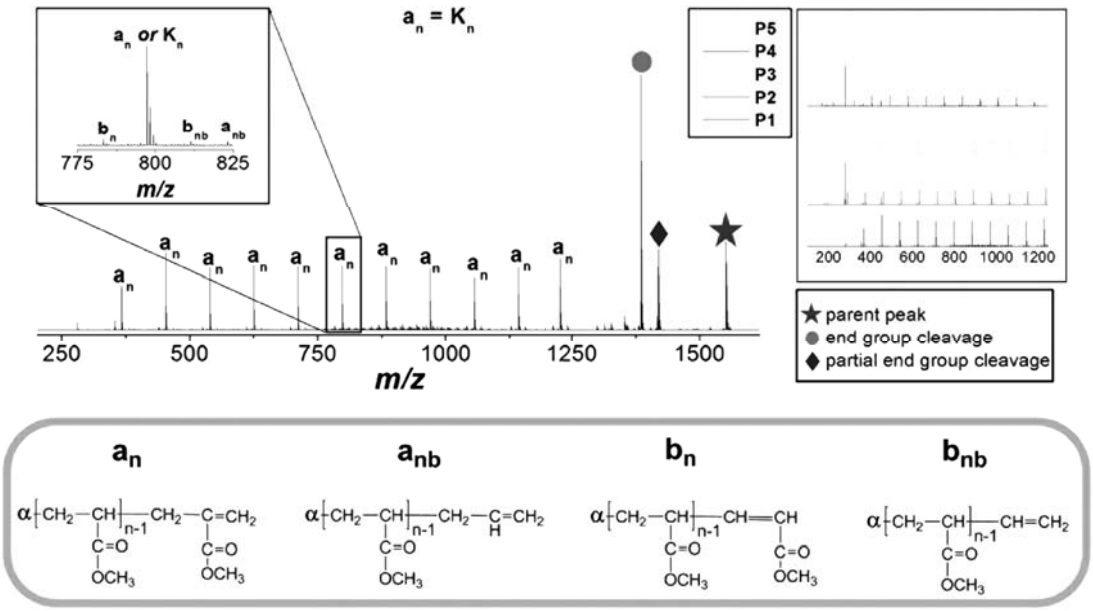
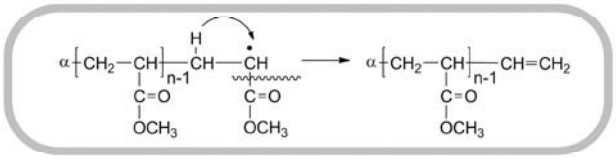


FIGURE 5 Fragmentation behavior in the lower molar mass range: assigned ESI-Q-TOF MS/MS spectrum of **P1** (left), comparison plot of **P1–P5** (right). Main fragment series corresponding to the proposed mechanisms.



SCHEME 3 Schematic representation of the possible fragmentation pathway for the fragment ion series $\mathbf{b_{nb}}$.

requires a lot more energy than dissociation via monomer evaporation. Therefore, $\mathbf{a_{nb}}$/$\mathbf{b_{nb}}$ series have very low relative abundances owing to the energetically more favorable depolymerization process. Owing to the fragile end-groups, fragment series with an intact ω end-group could not be observed in the tandem MS spectra. In addition, the comparison of the fragment signals in the low molar mass region of the investigated homopolymer species **P1–P5** yielded similar fragment series independent of the different end-groups, which is shown in Figure 5 (right spectrum, with the exception of polymer **P3**, some of the fragments of this polymer were radicalic species like $\mathbf{b_n}$•). Only for polymer **P1**, $\mathbf{a_n}$ species have exactly the same formula as the internal $\mathbf{K_n}$ species ($\mathbf{a_n} = \mathbf{K_n}$ for **polymer P1**). Therefore, it was not possible to decide whether the high abundance of these ion series was arisen from the $\mathbf{a_n}$ or $\mathbf{K_n}$ series for **P1**. For the rest of the polymers, it was achievable to distinguish $\mathbf{a_n}$ or $\mathbf{K_n}$ fragment ion series from each other and $\mathbf{a_n}$ series were the dominant fragment ion series in all tandem mass spectra.

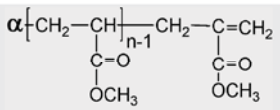
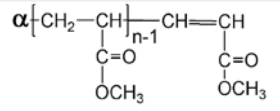
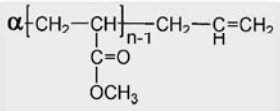
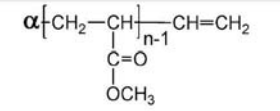
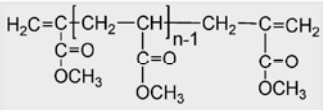
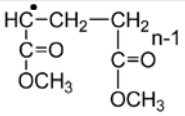
In the case of the homopolymers synthesized by the RAFT technique (**P1** and **P2**), the formation of an unsaturated fragment ion, which corresponds to the series $\mathbf{b_n}$, can be found in the MS/MS spectra owing to the cleavage of the RAFT end-group as a major fragmentation process. As possible mechanism behind this cleavage, the cyclic rearrangement via a four-membered transition state can be assumed, which resulted in a depolymerization reaction of the polymer chain to form the complete fragment ion series $\mathbf{b_n}$. Elimination of the RAFT end-group from the precursor ion would proceed via proton transfer from the last monomer unit to the one of the sulfur atoms in the terminal end-group, leading to the release of the ω end-group. The moderately high intensities of the end-group cleavage products (partial ◆ and complete •) can be a sign of the competition between the ω end-group cleavages in opposition to the backbone cleavages. All fragmentation products carry the α end-groups (with the exception of internal fragments such as $\mathbf{K_n}$ and $\mathbf{J_n}$), $\mathbf{a_n}$ series dominate in the whole mass spectra, whereas the analogous y_n series are not detectable. The reason for this behavior is the fragile ω end-groups can be cleaved off without any difficulty; after that, the fragmentation of primary $\mathbf{b_n}$ species results into $\mathbf{a_n}$ species through cyclic rearrangement mechanism via a four-membered transition state and the following

TABLE 1 Accurate Mass Measurements of PMA Product Ions Generated via End-Group Cleavages

Polymer	Parent Peak (*m/z*) $[SG(C_4C_2H_6)_{15}EG]^{Na+}$	Chemical Formula	End-Group Cleavages
P1	1,551.5471 *m/z* (15 RU[a])	SG[b]: $C_3O_2H_5$ EG[c]: $C_5S_3H_9$	($-C_5S_2H_9$) 133.01 *m/z* (partial EG cleavage) ($-HC_5S_3H_9$) 165.99 *m/z* (EG cleavage $\rightarrow b_{14}$) (further cleavage $\rightarrow a_{12}$)
P2	1,548.5908 *m/z* (15 RU)	SG: C_5H_8N EG: $C_7S_2H_5$	($-C_7H_5S$) 121.01 *m/z* (partial EG cleavage) ($-HC_7S_2H_5$) 153.99 *m/z* (EG cleavage $\rightarrow b_{14}$) (further cleavage $\rightarrow a_{12}$)
P3	1,694.7612 *m/z* (15 RU)	SG: $C_4H_7O_2$ EG: $C_{13}H_{29}PNO_4$	($-C_{13}H_{29}PNO_4$) 294.18 *m/z* (EG cleavage$\rightarrow b_{14}$•) (Homolytic C—ON bond cleavage) (Further cleavage via backbiting $\rightarrow a_{12}$)
P4	1,509.5400 *m/z* (15 RU)	SG: $C_6H_{11}O_2$ EG: Br	($-$HBr) 79.92 *m/z* ($\rightarrow b_{14}$) (Further cleavage $\rightarrow a_{12}$)
P5	1,435.5550 *m/z* (15 RU)	SG: $C_4H_7O_2$ EG: Cl	($-$HCl) 35.97 *m/z* ($\rightarrow b_{14}$) (Further cleavage $\rightarrow a_{12}$)

[a] RU: Repeating unit (MA: $C_4O_2H_6$, 86.04 *m/z*).
[b] SG: Starting group.
[c] EG: End-group.

TABLE 2 Structural Assignments for Sodiated Fragment Ions in the ESI-Q-TOF MS/MS Spectra

Fragmentation Product	Formula	Calculated *m/z*	Observed *m/z*	Error (ppm)
$\mathbf{a_n}$	 $[C_3O_2H_5(C_4O_2H_6)_{10}C_5O_2H_7]^{Na+}$	1,055.4306	1,055.4300	−0.56
$\mathbf{b_n}$	 $[C_3O_2H_5(C_4O_2H_6)_{10}C_4O_2H_5]^{Na+}$	1,041.4149	1,041.4110	−3.74
$\mathbf{a_{nb}}$	 $[C_3O_2H_5(C_4O_2H_6)_{11}C_3H_5]^{Na+}$	1,083.4619	1,083.4665	+4.25
$\mathbf{b_{nb}}$	 $[C_3O_2H_5(C_4O_2H_6)_{11}C_2H_3]^{Na+}$	1,069.4462	1,069.4372	−8.41
$\mathbf{K_n}$	 $[C_4O_2H_5(C_4O_2H_6)_1C_4O_2H_7]^{Na+}$	281.0996	281.0982	−4.98
$\mathbf{J_n\bullet}$	 $[C_7O_4H_{11}]^{Na+}$	182.0550	182.0561	+6.04

depolymerization reaction to create the complete fragment ion series $\mathbf{a_n}$. The details of the end-group cleavages are summarized for all investigated PMAs in Table 1, and the observed fragmentation product ions are provided in Table 2.

In CID analysis of **P3** (PMA obtained via NMP technique), it is again possible to show the loss of the ω end-group (•). For polymer **P3**, the proton transfer during the end-group cleavage was not observed like **P1** and **P2**. Homolytic dissociation of C—ON bonds at the labile terminating end-group requires less than half of the energy necessary to break backbone bonds or other rearrangement pathways. Therefore, the ω end-group can be cleaved off easily to create radicalic $\mathbf{b_n\bullet}$ fragment ion series. The radical $\mathbf{b_n\bullet}$ ions created via this way can further dissociate by backbiting rearrangements and β scissions to form terminal fragments with α end-groups and internal fragments with up to several repeating units. The backbiting pathway can convert radical $\mathbf{b_n\bullet}$ ions to more stable $\mathbf{a_n}$ species, and it can also give a rise to the internal fragments $\mathbf{K_n}$. For this polymer, $\mathbf{a_n}$ species are again the dominant species, but this time radicalic $\mathbf{b_n\bullet}$ fragment ion series have similar abundances to $\mathbf{a_n}$ species, and $\mathbf{b_n\bullet}$ species are detected only for NMP-generated PMA (**P3**). Once more, the depolymerization is more favorable compared to backbone cleavages and side group cleavages and therefore, $\mathbf{a_{nb}/b_{nb}}$ series have again extremely low relative abundances compared to the $\mathbf{b_n\bullet/a_n}$ species.

The tandem mass analysis of ATRP-generated polymers (**P4** and **P5**) provides fragmentation products with unsaturated chain ends owing to the decomposition of the ω end-group (•, the loss of HBr and HCl, dehalogenation). As it can be

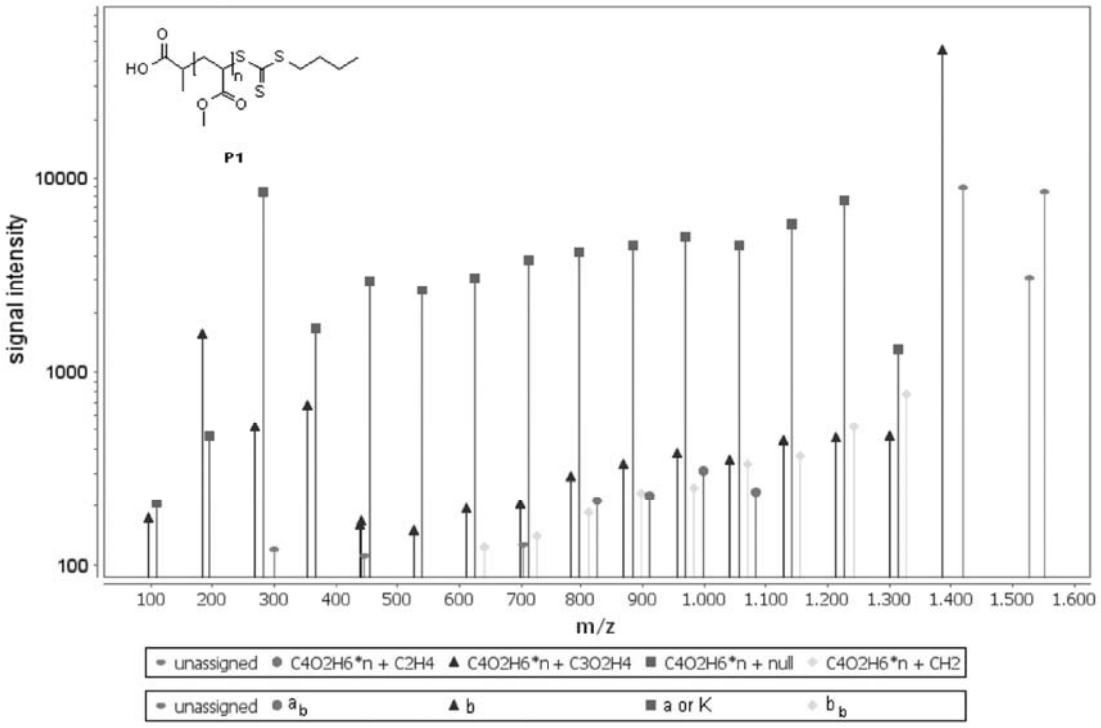


FIGURE 6 MALDI-TOF MS/MS spectrum of **P1**: evaluation by the new PLUMS software.

seen from the ESI-Q-TOF MS/MS spectra (Fig. 4), the bromine (**P4**) and the chlorine (**P5**) end-groups are easily cleaved off (HBr or HCl) under high energy conditions to form fragments. This behavior was already reported previously by several other groups.[6,23] Further cleavages in the upper molar mass region of **P4** and **P5** occurred owing to CH_3Br and CH_3Cl losses (▼). It was again possible to observe the same fragmentation products ($\mathbf{a_n}$, $\mathbf{b_n}$, $\mathbf{a_{nb}}$, and $\mathbf{b_{nb}}$) for these ATRP-generated polymers.

In this contribution, other than the application of tandem mass analysis of CRP-generated polymers via various ionization techniques for investigating the differences in fragmentation patterns, we would like to present the automated evaluation of tandem mass spectra of homopolymers by utilizing the PLUMS software for a faster and easier analysis (http://bio.informatik.uni-jena.de/software/plums).[21] PLUMS is an interpretation software that analyzes tandem mass spectra of synthetic polymers. The software assists to identify fragmentation series of homopolymers by determining sum formula for the repeating unit and start- as well as end-groups of the fragments. Further knowledge of the polymer class or the fragmentation mechanism is not required. The software user simply requires a peak list of the tandem MS spectrum as a XML file. As a result, a list of possible molecular formula of each fragment and the mass error to the calculated mass is provided. The user can decide on the correct molecular formula and, finally, can see the annotated spectrum from the specified information. The usage of the PLUMS software for the interpretation of **P1** is shown exemplarily in Figure 6. Applying a mass error of 0.5 *m/z* units for the MALDI-TOF MS/MS data, the software identifies five different fragment series with an appropriate end-group formula. Comparing the manual and the automated evaluation output, it is clearly visible that both interpretation methods delivered the same results (black box, Fig. 6). We can conclude from these findings that this new software tool represents an important assistant in accomplishing the interpretation of tandem mass spectra of homopolymers in a fast and automated manner.

With this approach, it is possible to gain further insights into the polymer composition by straightforward and rapid interpretation of tandem mass spectra. This kind of software development studies for synthetic polymers will certainly increase the usage of MS-based approaches to characterize synthetic polymers, as already seen in the fields of proteomics, metabolomics, genomics, and glycomics.

CONCLUSIONS

The analysis of synthetic polymer samples presents special challenges and limitations because of its complex structure. MS-based techniques can provide solutions for the polymer science community. To be able to gain insights of the macromolecular structure with MS, it is important to develop special data interpretation software tools to identify fragmentation products obtained by MS/MS as in the fields of various "-omics." In the first part of this study, a systematic and experimental comparison of three soft ionization methods (ESI, APCI, and MALDI) was presented to analyze PMAs prepared by different CRP techniques. ESI and APCI are better suited

for the analysis of the end-group functionalities of CRP-prepared polymers; however, MALDI is superior to obtain molar mass information about these polymers. As a consequence, all MS techniques are complementary to gain information about CRP polymerizations. In the second part, fragmentation pathways of the PMAs with fragile end-groups obtained from CRP techniques were investigated comprehensively to identify differences. As a final part, the evaluation of the tandem mass spectra with a dedicated special software was demonstrated to show that this kind of software will be helpful for polymer scientists in the future for a computerized analysis of complicated tandem mass data. The future challenge will be focused on the expansion of this software including the interpretation of tandem mass spectra from block and random copolymers with a sequence analysis (*de novo* sequencing for polymers).

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Publication P6:

Detailed characterization of poly(2-oxazoline)s by energy-variable collision-induced dissociation study

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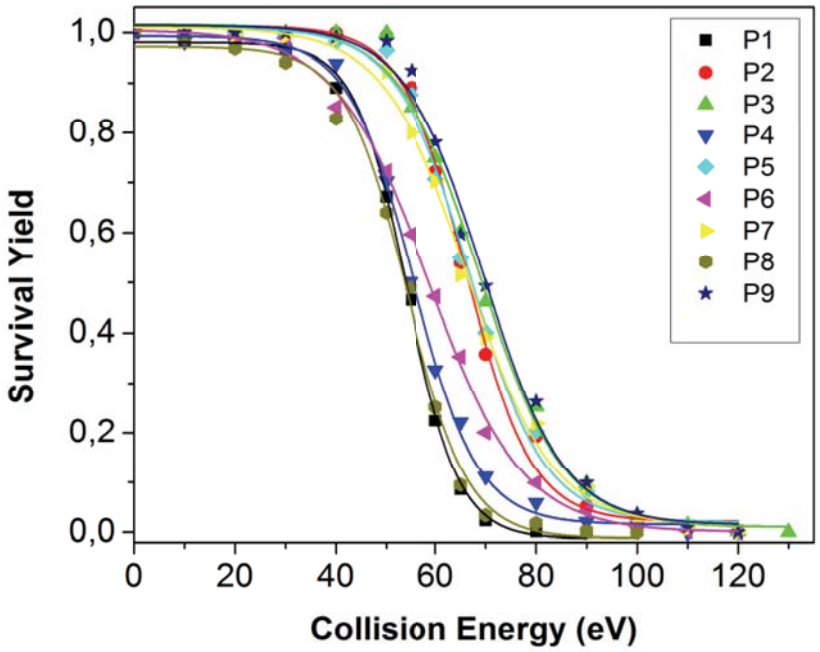

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Detailed characterization of poly(2-ethyl-2oxazoline)s by energy variable collision-induced dissociation study

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RATIONALE: Poly(2-oxazoline)s are important polymers and can be considered as pseudo-peptides which makes them important for biomedical and life science applications. This prompts the need for a detailed characterization of these polymers via different analytical tools such as mass spectrometry. Here, the energy-variable collision-induced dissociation (CID) of poly(2-ethyl-2-oxazoline)s was studied by electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-QTOFMS/MS) to gain further structural information about this polymer type.
METHODS: All polymers were analyzed using manual flow injection of samples into an ESI-QTOF mass spectrometer. Mass spectra (MS and MS/MS) were obtained in the positive ion mode over a mass-to-charge (m/z) range from 50 to 3000.
RESULTS: The dependency of the fragmentation patterns as a function of collision energy was examined and the characteristic collision energy (CCE or CE_{50}) values for various poly(2-ethyl-2-oxazoline)s with different end-groups were calculated. The effect of molar masses on the CCE values was investigated via the survival yield (SY) method and a linear relationship between CCE values and the degree of polymerization for the PEtOx polymers was observed.
CONCLUSIONS: This study showed that ESI-MS/MS is very useful for differentiating poly(2-ethyl-2-oxazoline)s with different end-groups by varying the collision energy. The SY method has the potential to determine the importance of the end-groups on the fragmentation behavior of this polymer type. Copyright © 2013 John Wiley & Sons, Ltd.

Tandem mass spectrometry (MS^n or MS/MS) has been proven to be a powerful tool for the structural characterization of synthetic polymers.[1–4] It represents an important technique to complement the existing analytical methods (e.g. separation systems) that face the challenge of complexity and diversity of synthetic polymers. MS^n experiments can provide structural information and help to understand the fragmentation mechanisms of different homopolymer classes. However, there is still a need for a methodology to handle sample complexity within this field of research. One approach is to develop standardized tandem mass spectrometry methods that allow structural information of synthetic polymers to be obtained from the fragmentation patterns. In order to accomplish this task an understanding of the fragmentation behavior of synthetic polymers is required in different excitation conditions and as a function of their composition. This prompts the need for the analysis of polymers via energy-dependent collision-induced dissociation (CID) experiments to investigate the dependency of the fragmentation patterns as a function of collision energy.

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Electrospray ionization mass spectrometry (ESI-MS) is a very sensitive and accurate soft ionization technique,[5] suitable for the study of many synthetic polymers with minimal sample consumption. Moreover, it generally allows the ionization of different macromolecules with little or no fragmentation. Hence, ESI-MS/MS can be employed for the investigation of the amount of energy necessary to drive the reaction fast enough to observe product ions, and to calculate characteristic collision energy (CCE or CE_{50}) values for synthetic polymers. The CCE needed to obtain 50% fragmentation has already been used as a tool to distinguish different classes of polymers such as polyethers, polymethacrylates, polyesters, and polysaccharides.[6–9] Obtaining survival yield (SY) curves from the energy-dependent ESI-MS/MS data provides a useful way of presenting all the information on the CID processes undergone by a specific type of polymer ion. SY analysis is used in mass spectrometry as a tool for evaluating precursor ion stability and internal energy. CCE (CE_{50}) values can be determined easily from the SY curves and these values could discriminate between different compounds (even structural and positional isomers). The SY method reveals insights about the energy requirements of the fragmentation pathways of polymers. Therefore, it is a promising way for identification and discrimination of polymers with different functionalities or backbones.

Poly(2-ethyl-2-oxazoline)s (PEtOx) are an important subclass of polymers that recently has gained significant attention, e.g. for various applications as bio-inspired "smart"

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materials.[10–12] They offer biocompatibility as well as the stealth effect (i.e. they are not recognized by the human body and thus are suitable for drug delivery applications). As a consequence, this material class has been considered as an alternative for the widely applied poly(ethylene glycol) (PEG) in the field of modern drug delivery.[13,14] In order to utilize PEtOx for these applications, the PEtOx polymers have to be characterized comprehensively using several different analysis techniques. MS-based techniques can provide significant structural information and, in particular, detailed information can be derived from energy-dependent MS/MS techniques. In the present study, the energy-dependent CID of sodiated PEtOxs with different functional end-groups was studied by ESI quadrupole time-of-flight (QTOF) MS/MS, and the fragmentation properties of PEtOxs were interpreted using the survival yield (SY) method. The data obtained will provide crucial information for the analysis of PEtOxs in the future.

EXPERIMENTAL

Materials

2-Ethyl-2-oxazoline (EtOx; 99%; Acros Organics, Geel, Belgium) was dried over barium oxide and distilled under argon prior to use. Methyl *p*-toluenesulfonate, propargyl *p*-toluenesulfonate, allyl *p*-toluenesulfonate and *p*-toluenesulfonic acid were obtained from Sigma Aldrich (Oakville, ON, Canada), distilled to dryness and stored under argon. Methacrylic acid (MAA), 9-(chloromethyl)anthracene, anthracene-9-carboxylic acid, *t*-butyl 1-piperazine-carboxylate and sodium azide were purchased from Sigma Aldrich and used as received. Triethylamine (NEt_3) was dried over potassium hydroxide and distilled under argon. Acetonitrile and dichloromethane were obtained from Acros Organics (extra dry). The solvents used for the ESI-QTO FMS measurements were LC/MS grade (Sigma-Aldrich) and were used as received.

General methods and instrumentation

An Initiator Sixty single-mode microwave synthesizer from Biotage (Uppsala, Sweden), equipped with a noninvasive IR sensor (accuracy: ±2%), was used for polymerizations under microwave irradiation. Microwave vials were heated overnight to 110 °C and allowed to cool to room temperature under argon before use. All polymerizations were carried out with temperature control. Size- exclusion chromatography (SEC) measurements were performed on a Shimadzu (Duisburg, Germany) system equipped with a SCL-10A system controller, a LC-10 AD pump, a RID-10A refractive index detector, a SPD-10A UV detector at 254 nm and a PLgel 5 µm Mixed-D column at 50 °C utilizing a chloroform: triethylamine:2-propanol (94:4:2) mixture as eluent at a flow rate of 1 mL/min. ^{1}H NMR spectra were recorded on a Bruker AC 300 MHz spectrometer (Karlsruhe, Germany) at room temperature, with $CDCl_3$ as solvent.

Synthesis of poly(2-ethyl-2-oxazoline)s

The PEtOx samples were prepared according to published synthetic protocols.[4,15–19] A stock solution containing initiator, monomer, and solvent was prepared. The monomer concentration was adjusted to 4 M. The vials were heated to 140 °C for a predetermined time in the microwave synthesizer. After the solution had been allowed to cool, the respective terminating agent was added in excess and stirred at ambient or elevated temperature overnight. Subsequently, the polymer solution was washed three times with water, brine and dried over $MgSO_4$. After the solution had been concentrated under reduced pressure the polymer was precipitated in ice-cold diethyl ether yielding a whitish to slightly yellowish product. ^{1}H-NMR and SEC samples were analyzed to determine the monomer conversion, end-group functionality and the molar masses of the polymers, respectively.

ESI-QTOF MS and MS/MS measurements

All compounds were analyzed using a micrOTOF Q-II (Bruker Daltonics, Bremen, Germany) quadrupole time-of-flight mass spectrometer equipped with an automatic syringe pump from KD Scientific (Holliston, MA, USA) for sample injection. The mass spectrometer was run at a capillary voltage of 4.5 kV and a desolvation temperature of 180 °C, and operated in positive ion ESI mode. Nitrogen was used as the nebulizer and drying gas. For the CID experiments, the quadrupole mass analyzer was used for selection of the precursor ions and nitrogen was used as the collision gas. The concentration of the samples was 10 µg/mL and all samples were injected using a constant flow rate (180 µL/h) of sample solution. The solvent was a chloroform:acetonitrile (10:90, v/v) mixture. There was no salt or acid addition prior to analysis, but ionization occurred readily as a result of the sodium content naturally present in the glass or in the polymer sample. A total of 60 scans/spectrum were averaged and the quoted *m/z* values are monoisotopic. The ESI-QTOF MS instrument was calibrated in the *m/z* range 50 to 3000 using an internal calibration standard (Tunemix solution), supplied by Agilent Technologies (Böblingen, Germany). All data were processed via Bruker Daltonics Data Analysis software version 4.0.

The SY curve was determined by fragmenting the sodiated PEtOx polymers starting at a collision energy (CE) of 10 eV and increasing the CE until the peak for the precursor ion was no longer detectable. The MS/MS spectrum for each collision energy value was used to determine the experimental SY, as the precursor ion intensity divided by the sum of the precursor and product ion intensities. The CCE (CE_{50}) values were estimated by a sigmoidal fit of the SY curve.[20]

RESULTS AND DISCUSSION

Poly(2-oxazoline)s (POxs) have been studied previously by mass spectrometry using a variety of ionization techniques such as ESI and matrix-assisted laser desorption/ionization (MALDI), and the fragmentation mechanisms of electrospray-generated POx ions were examined in our earlier work.[3] In the present contribution, the main aim was to study the detailed fragmentation patterns of various PEtOx with different end-groups using ESI-QTO FMS/MS by varying the applied collision energy values (0 to 200 eV) and to create SY curves from the data obtained via these energy-dependent CID experiments. For this purpose, the livingness of the cationic ring-opening polymerization

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(CROP) of EtOx was exploited by the utilization of various functional initiators and end-capping agents. As displayed in Scheme 1, the resulting PEtOx polymers covered a wide range of functional groups, including hydrogen, methyl, allyl, propargyl, anthracenyl, or protected sugar moieties at the initiating (α) chain ends, and hydroxyl groups, esters or amines at the terminating (ω) chain ends. The sodiated molecule of each PEtOx polymer sample was exposed to increasing collision energy, and $[M+Na]^+$ ion intensity decreased in a sigmoidal way.

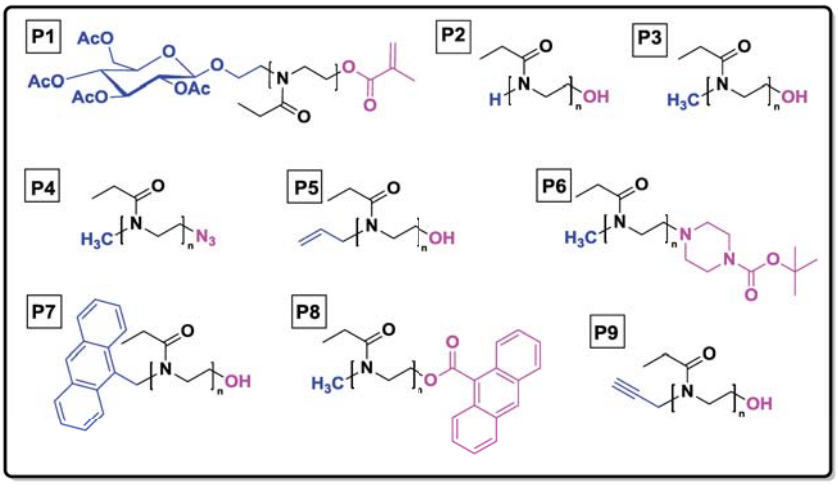
The sugar-decorated macromolecular structure **P1** was selected as a representative example to explain the details of this study. Figure 1 demonstrates the fragmentation behavior of sodiated **P1** under different collision energy values (10 to 100 eV). As can be readily seen from the ESI tandem mass spectra of sodiated **P1**, the loss of the methacrylate end-group can be observed starting at a collision energy of 40 eV. Until this point, no distinct fragmentation was visible. Subsequent to the end-group cleavage, a depolymerization mechanism started, leading to the formation of stable cationic oxazolinium species. The signal intensity of product ions that result from the depolymerization mechanism increased gradually upon increasing the collision energy until at 90 eV only the protected sugar carrying one oxazolinium remained (*m/z* 474.19). The proposed depolymerization mechanism is shown for the representative example, sodiated **P1**, in Scheme 2.

The fragmentation mechanism was investigated by varying the collision energy in CID experiments for the $[M+Na]^+$ ions for all PEtOxs (**P1–P9**). PEtOxs with a fragile end-group, such as an ester, can be fragmented readily by a depolymerization process. At the beginning of the fragmentation process, the loss of the labile end-group was observed with consecutive loss of monomer units (unzipping or depolymerization mechanism). Upon increase of the collision energy, this depolymerization mechanism could be easily followed down to one monomer unit with the initiating end-group. On the other hand, for PEtOxs with poor leaving end-groups such as a hydroxyl, a large number of further product ion series can be observed due to other types of backbone cleavages (e.g. 1,4 hydrogen or ethylene elimination from the backbone, McLafferty rearrangement, and side- group eliminations).[3,16] For the latter PEtOxs, the depolymerization mechanism was not the main fragmentation pathway. These findings revealed that the fragmentation mechanism was mainly influenced by how good the ω end-group was as a leaving group. On the other hand, the influence of the α end-groups on the fragmentation mechanism was less pronounced, supporting the hypothesis that depolymerization is observed for good leaving groups, which can easily form a stable oxazolinium end-group.

A plot of the survival yield (SY) curves of various sizes of the studied PEtOxs allowed the collision energy values related to the characteristic 50% point of the $[M+Na]^+$ precursor ion to be determined. Figure 2 shows the suggested explanation of this process for the glucose-functionalized PEtOx (**P1**) with 10 monomer units. The experimental data points are shown together with the corresponding sigmoidal fits. The abscissa of the characteristic point at 50% SY of the sigmoidal fit represents the CCE (CE_{50}) value for the investigated PEtOx (**P1**).

Figure 3 displays the SY curves for the $[M+Na]^+$ precursor ions corresponding to PEtOx chains with different degrees of polymerization (DP) that are present in **P1**. The calculated CCE (CE_{50}) values of PEtOxs, as a function of the precursor ion mass, showed a relationship between the CCE (CE_{50}) values and the molecular size for all PEtOx polymers. A lower collision energy was required to induce fragmentation for shorter polymer chains, and it was shown that the collision energy required to achieve 50% fragmentation (CCE or CE_{50}) was linearly dependent on the precursor ion mass (i.e., the number of the repeat units, on the size, or on the number of degrees of freedom (DoF)). This observation correlated well with recent findings for various other synthetic and natural polymers such as polyethers, polymethacrylates, polyesters, and polysaccharides.[6,8]

The collision energy necessary to promote fragmentation was also investigated for PEtOx with different end-groups (**P1–P9**). Comparison of the SY curves obtained (Fig. 4) showed that one group of polymers apparently required more energy to undergo fragmentation (CCE > 66 eV): **P2**, **P3**, **P5**, **P7** and **P9**. All these PEtOxs contain a hydroxyl ω end-group and fragment through backbone cleavages (e.g. 1,4 hydrogen or ethylene elimination, McLafferty rearrangement,



Scheme 1. Schematic representation of the structures of the studied poly(2-ethyl-2-oxazoline)s with different end-groups.

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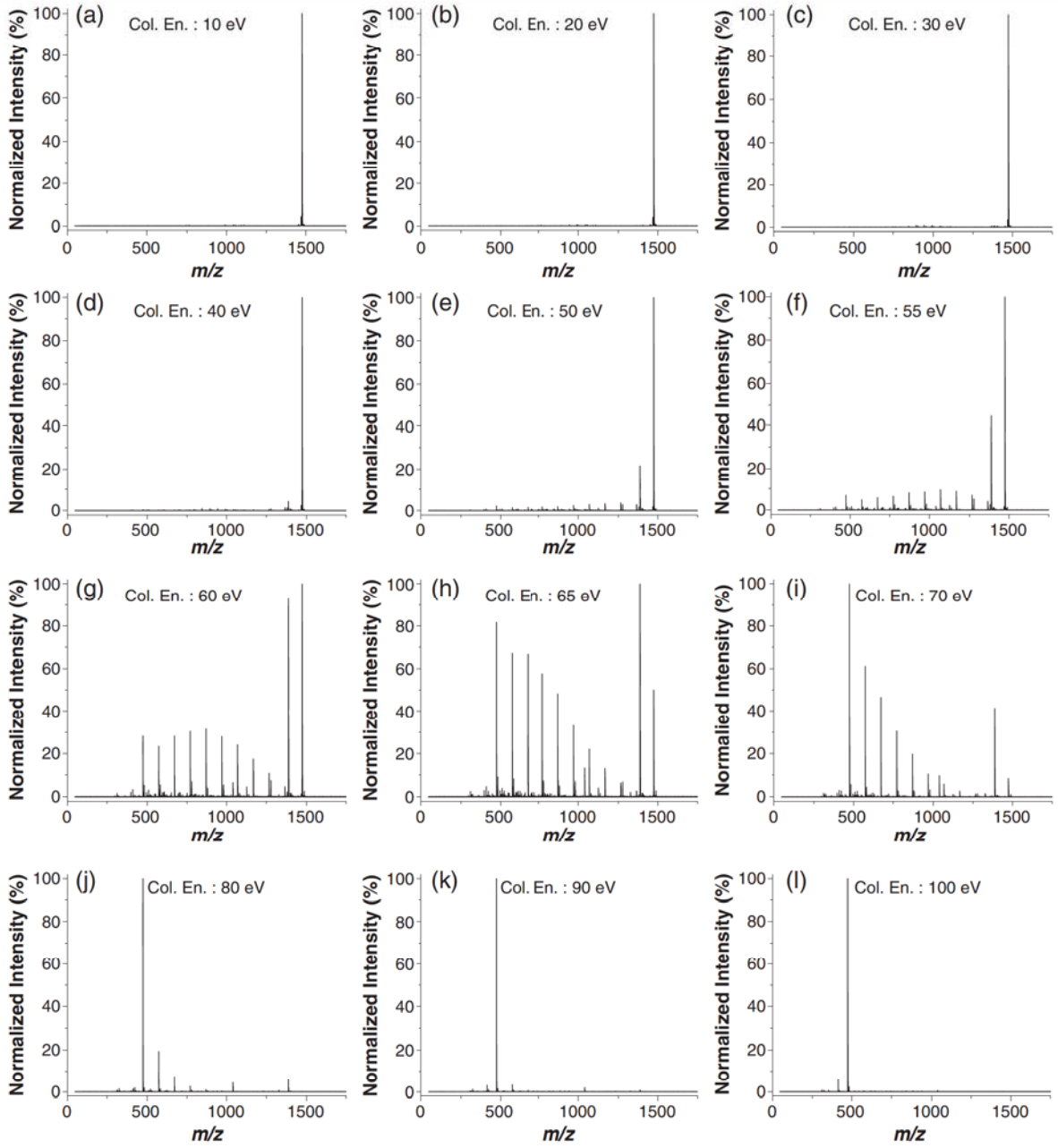
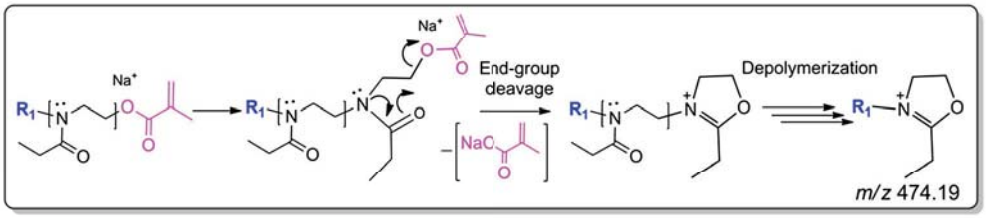


Figure 1. ESI-MS/MS spectra of sodiated PEtOx (**P1**) at *m/z* 1473.82 recorded at different collision energies (10 to 100 eV).



Scheme 2. Schematic representation of the possible depolymerization mechanism for the formation of product ions from the poly(2-ethyl-2-oxazoline)s with fragile terminating end-groups (representative example: **P1**).

and side group eliminations) instead of the depolymerization mechanism. Among the four other polymers **P1** and **P8** have the lowest CCE values. They have ester ω end-groups, which promote fragmentation via the depolymerization mechanism. Thus, the varying ω end-groups can be categorized according to their CCE values and bond energies: ester

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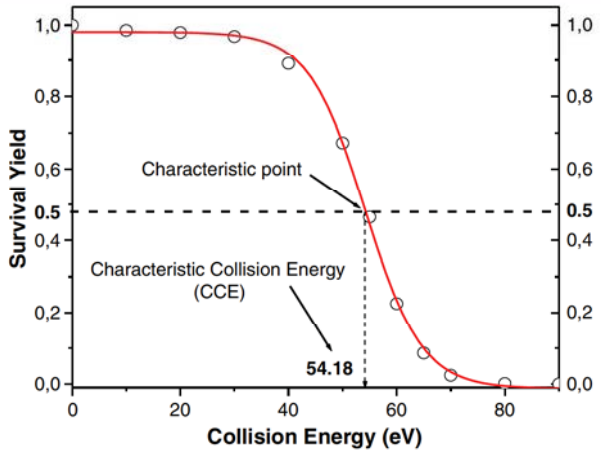
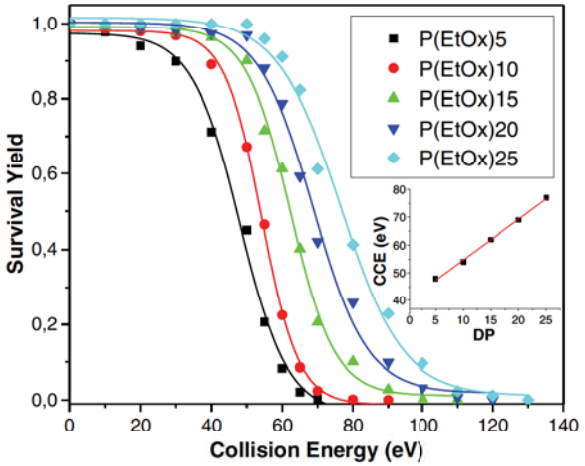


Figure 2. Survival yield (SY) curve for sodiated glucose-functionalized PEtOx with 10 monomer units (**P1**). The CCE value was defined as the abscissa of the characteristic point at 50% SY of the sigmoidal fit.

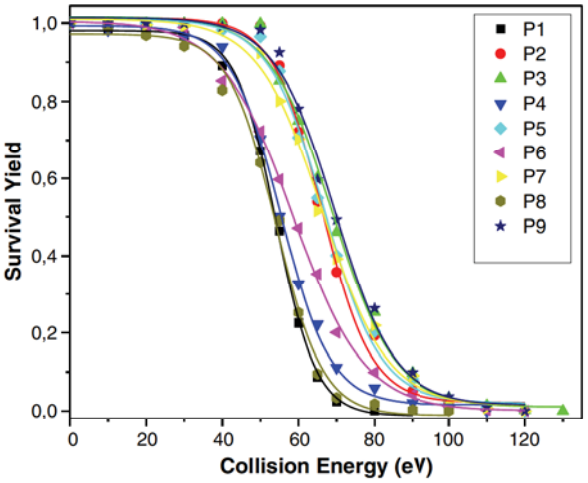
azide < amine < < hydroxyl. In turn, the α end-group of the studied PEtOx polymers has no significant influence on the CCE value since all polymers with hydroxyl end-groups have similar CCE values, despite the structural diversity of their α end-groups. However, the influence of the end-group on the CCE is still less significant than the mass of the precursor ion.

The data obtained suggest that fragile end-groups fragment most easily (low CCEs), whereas poor leaving end-groups require higher collision energies to promote fragmentation among the different end-groups for this polymer class. As explained above, this is a direct result of the different types of fragmentation pathways induced by the attached ω end-group: in the case of PEtOxs with labile end-groups, fragmentation mechanism involving a depolymerization mechanism requires a lower energy for fragmentation. On the other hand, PEtOxs with poor leaving end-groups need higher collision energies to fragment *via* backbone cleavages. Additional research (i.e. further experiments and theoretical calculations) would be necessary to confirm this behavior. Overall, the results obtained from



Polymer	CCE (eV)
PEtOx5	48.01
PEtOx10	54.18
PEtOx15	62.26
PEtOx20	68.68
PEtOx25	76.49

Figure 3. Survival yield curves for different molar masses of sodiated **P1** (PEtOx polymer with DP = 5, 10, 15, 20 and 25, respectively).



Polymer	CCE (eV)
P1	54.17
P2	66.20
P3	68.87
P4	55.41
P5	66.86
P6	58.56
P7	66.39
P8	54.10
P9	69.57

Figure 4. Survival yield curves of various sodiated PEtOx species with different end-groups (**P1–P9**).

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this study indicate the utility of SY curves as a tool to correlate the macromolecular structure of PEtOxs with their fragmentation behavior. Both the DP of the precursor ion and the nature of the ω end-group were found to affect the energy required to induce fragmentation via MS/MS, whereas the α end-groups had no significant effect on the CCE values.

CONCLUSIONS

Important information can be obtained from MS^n experiments by applying varying collision energies to poly-(2-ethyl-2-oxazoline) samples. The data acquired via energy-dependent CID experiments was utilized to obtain SY curves. From these SY curves, the collision energies necessary to achieve 50% fragmentation (CCE or CE_{50} values) were obtained, allowing these values to be correlated with the size of the polymers and the end-groups of PEtOxs. The results obtained suggest that the CCE values could help to understand fragmentation mechanisms and that they could be used for compound identification and discrimination. The terminating (ω) end-groups were found to have an effect on the fragmentation pathways, because these groups have a crucial role in the fragmentation mechanism. Further investigations would be necessary to confirm this behavior.

Having established this generally applicable method to characterize these polymers, future work will focus on the investigation of the POxs with different side groups such as poly(2-methyl-2-oxazoline), poly(2-(1-ethylpentyl)-2-oxazoline), poly(2-phenyl-2-oxazoline), poly(2-(2,6-difluorophenyl)-2-oxazoline) and poly(2-p-terbutylphenyl-2-oxazoline). Investigating more complex POxs systems (i.e. block, graft, and random copolymers as well as star-shaped polymers) via applying varying collision energy values would also be of interest. Such studies would facilitate the construction of a MS^n product ion library of poly(2-oxazoline)s including CCE data for an effective analysis and discrimination of homopolymers and copolymers with different end and side groups of this type of synthetic polymer.

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Publication P7:

Comparison of ESI, APCI and MALDI for the tandem mass analysis of poly(2-ethyl-2-oxazoline)s with different end-groups

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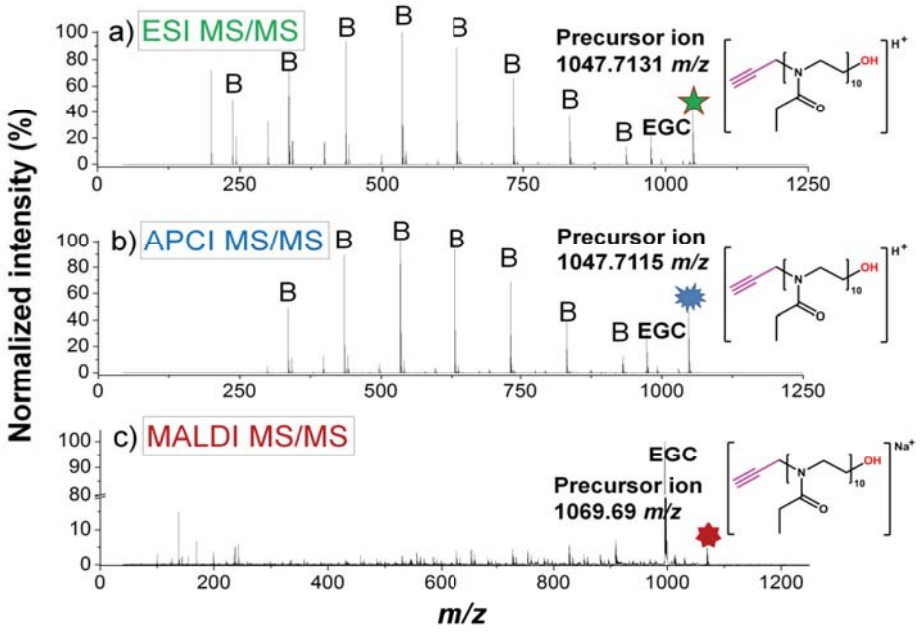
Esra Altuntaş, Christine Weber, Kristian Kempe, and Ulrich S. Schubert

European Polymer Journal **2013**, *in press.*



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Comparison of ESI, APCI and MALDI for the (tandem) mass analysis of poly(2-ethyl-2-oxazoline)s with various end-groups

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ABSTRACT

A series of poly(2-ethyl-2-oxazoline) (PEtOxs) with various end-groups were analyzed by different mass spectrometry (MS) techniques (electrospray ionization mass spectrometry (ESI-MS), atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)). In a systematic comparison study the three soft ionization methods (ESI, APCI and MALDI) were exploited to investigate the PEtOxs with regard to their molecular composition, molar masses (M_n and M_w) and polydispersity index (PDI) values. In addition, a detailed characterization *via* tandem mass spectrometry experiments was performed to gain further structural information about these polymers and their fragmentation patterns. The effect of the end-groups on the fragmentation patterns was investigated for this polymer type. Additionally, the successful interpretation of tandem MS data *via* a special software was shown for this type of polymer in order to open up this field also for polymer scientists who are not MS-specialists. The insights gained from this work will assist the structural elucidation of a broad range of synthetic polymer classes in the future.

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1. Introduction

Advanced mass spectrometry (MS) techniques have been used for many different fields such as proteomics, metabolomics and lipidomics for a long time [1–4]. The development of living and controlled polymerizations has given a rise to manifold synthetic polymeric materials with a large diversity in architectures and end-groups. As a result, MS techniques have been increasingly utilized for the characterization of synthetic polymers to provide structural insights to understand the molecular complexity of polymers [5–7]. Suitable MS techniques for the characterization of synthetic polymers must be based on soft ionization techniques such as in electrospray ionization mass spectrometry (ESI-MS) [8], atmospheric pressure chemical ionization mass spectrometry (APCI-MS) [9] and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) [10,11]. In addition to the determination of molar masses (M_n and M_w), the polydispersity index (PDI) and the end-groups of the polymer by simple MS, tandem MS (MS/MS) can be applied in order to verify the peak assignments and to identify individual end-groups, to differentiate isobaric and isomeric species and to analyze the macromolecular architectures of polymers in detail. Tandem mass spectrometry experiments are performed upon certain precursor ions of interest in order to gain further structural information from the resulting fragmentation products and to understand the fragmentation mechanism of the investigated polymer class [12]. A characteristic fragmentation pattern can be defined for a certain type of synthetic polymer and used as a reference to characterize

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unidentified polymers within the same class. MALDI and ESI are often used to characterize polymers *via* MS and MS/MS, but APCI is less frequently applied for synthetic polymers. However, the APCI technique coupled to liquid chromatography (LC) methods open new opportunities for the characterization of polymers. APCI works very well, in particular for low molar mass polymers that are poorly ionizable by the usual methods due to their apolar character [13].

Poly(2-oxazoline)s (POx) are an important class of polymers with a wide range of possible applications owed to their structural diversity. POx can be considered as pseudo-peptides due to their structural relationship to polypeptides which makes them highly interesting for biomedical and life science applications [14–16]. Bio-inspired "smart" materials and their solution properties can be fine-tuned by varying functional groups, *i.e.* end-groups and the side-chains of this type of polymer. Therefore, it is essential to synthesize these polymers with various end-groups to satisfy the need for different applications. The living cationic ring-opening polymerization (CROP) of 2-oxazolines provides easy access to various well-defined POx, whereby the end-group functionality can be controlled during initiation and termination, and side-chain functionalities can be introduced by the copolymerization with functional monomers [17–19].

Noteworthy in view of biomedical applications is the biocompatibility of certain poly(2-oxazoline)s, namely poly(2-methyl-2-oxazoline) (PMeOx) and poly(2-ethyl-2-oxazoline) (PEtOx) and their copolymers [20,21]. Both water soluble polymers are considered as alternative systems to commonly used poly(ethylene glycol) [22]. The similarity in their properties in combination with the simple fine-tuning of the hydrophilic/hydrophobic balance of the POxs by the variation of the side chains make them highly interesting for biomedical application, such as drug delivery systems, and polymer therapeutics [23].

For biomedical applications of PEtOx, a well-defined and pure material has to be synthesized in a controlled way and a detailed characterization of these polymers has to be performed in order to draw conclusive structure–property relations. For this purpose, different (tandem) mass spectrometry (MS and MS/MS) techniques were recently utilized to obtain information about their molar masses in addition to potential side reactions [24–26]. Schubert and coworkers have already investigated the fragmentation mechanism of POxs with different side groups (*i.e.* methyl, ethyl, 1-ethylpentyl, phenyl, *o*-difluorophenyl and *p*-*tert*-butylphenyl) in order to explore the effect of the side groups on the fragmentation mechanisms [25]. In this contribution, our aim is to analyze PEtOxs with different functional end-groups using different (tandem) mass spectrometry techniques such as ESI-MS, APCI-MS and MALDI-MS and tandem mass spectrometry (MS/MS). It is important to compare these three soft ionization methods (ESI, APCI and MALDI) for the analysis of polymers in a systematic manner to gain a deeper insight of their ionization behaviors. To the best of our knowledge, no detailed comparison of these three techniques has been reported so far for synthetic polymers. The detailed characterization of PEtOx *via* different mass spectrometry techniques, their fragmentation mechanisms, and the application of a special software for the interpretation of tandem MS data of synthetic polymers is presented. The PLUMS software was used for the first time to analyze ESI and APCI MS/MS data.

2. Experimental

2.1. Materials

2-Ethyl-2-oxazoline, methyl *p*-toluenesulfonate, propargyl *p*-toluenesulfonate, allyl *p*-toluenesulfonate and *p*-toluenesulfonic acid were obtained from Aldrich and were distilled to dryness and stored under argon. 9-(Chloromethyl)-anthracene, anthracene-9-carboxylic acid, *t*-butyl 1-piperazine-carboxylate and sodium azide were purchased from Aldrich and used as received. Acetonitrile and dichloromethane were obtained from Acros. Solvents used for the ESI-Q-TOF and APCI-Q-TOF MS measurements were LC-MS grade solvents and were purchased from Sigma Aldrich (used as received).

2.2. General methods and instrumentation

The Initiator Sixty single-mode microwave synthesizer from Biotage, equipped with a noninvasive IR sensor (accuracy: ±2%), was used for polymerizations under microwave irradiation. Microwave vials were heated overnight to 110 °C and allowed to cool to room temperature under argon before usage. All polymerizations were carried out with temperature control. Size exclusion chromatography (SEC) measurements were performed on a Shimadzu system equipped with a SCL-10A system controller, a LC-10AD pump, a RID-10A refractive index detector, a SPD-10A UV-detector at 254 nm and a PLgel 5 μm Mixed-D column at 50 °C utilizing a chloroform:triethylamine:2-propanol (94:4:2) mixture as eluent at a flow rate of 1 mL/min. ^{1}H NMR spectra were recorded on a Bruker AC 300 MHz spectrometer at room temperature, with $CDCl_3$ as the solvent. ESI-Q-TOF and APCI-Q-TOF MS measurements were performed with a micrOTOF Q-II (Bruker Daltonics) mass spectrometer from Bruker Daltonics (Bremen, Germany), equipped with either an electrospray ionization (ESI) or an atmospheric pressure chemical ionization (APCI) interface. MALDI-TOF MS experiments were performed with an Ultraflex III TOF/TOF (Bruker Daltonics, Bremen, Germany) equipped with a Nd:YAG laser and a collision cell. Agilent 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) was used for the APCI-MS experiments to obtain better signal intensities.

2.3. Synthesis

2.3.1. General procedure for MeOTs initiated poly(2-ethyl-2-oxazoline)s

A stock solution containing initiator (methyl *p*-toluenesulfonate), monomer (2-ethyl-2-oxazoline) and solvent (acetonitrile) was prepared. The monomer concentration was adjusted to 4 M and a monomer to initiator ratio of 10 and 20 was targeted, yielding a molar mass of about 1000 and 2000 g/mol, respectively. The vials were heated to 140 °C for a predetermined time in the microwave

synthesizer. After cooling, the respective terminating agent was added in excess and stirred at ambient (sodium azide, *t*-butyl 1-piperazine-carboxylate/potassium carbonate) or elevated temperature (water, anthracene-9-carboxylic acid/triethylamine) overnight. Subsequently, the polymer solution was washed three times with water, brine and dried over $MgSO_4$. After concentrating the solution under reduced pressure the polymer was precipitated in ice-cold diethyl ether yielding a whitish to slightly yellowish product. ^{1}H NMR and SEC samples were prepared to determine the monomer conversion and the molar masses of the polymer, respectively.

2.3.2. General procedure for poly(2-ethyl-2-oxazoline)s terminated with water

A stock solution containing initiator (propargyl *p*-toluenesulfonate, allyl *p*-toluenesulfonate or *p*-toluenesulfonic acid), monomer (2-ethyl-2-oxazoline) and solvent (acetonitrile) was prepared. In case of using 9-(chloromethyl)-anthracene as initiator the solvent was changed to dichloromethane. The monomer concentration was adjusted to 4 M and a monomer to initiator ratio of 10 and 20 was targeted, yielding a molar mass of about 1000 and 2000 g/mol, respectively. The vials were heated to 140 °C for a predetermined time in the microwave synthesizer. After cooling, the reaction was quenched by the addition of 50 μL water. ^{1}H NMR and SEC samples were prepared to determine the monomer conversion and the molar masses of the polymer, respectively.

2.4. Mass spectrometry measurements

2.4.1. ESI-Q-TOF-MS and MS/MS measurements

PEtOx samples were analyzed by using a micrOTOF Q-II (Bruker Daltonics) mass spectrometer equipped with an automatic syringe pump from KD Scientific for sample injection. The ESI-Q-TOF mass spectrometer was running at 4.5 kV, at a desolvation temperature of 180 °C. The mass spectrometer was operating in the positive ion mode. Nitrogen was used as the nebulizer and drying gas. For the CID experiments, a quadrupole was used for selection of the precursor ions and argon was used as the collision gas. The collision energy was set according to the MS/MS experiments to be carried out by using the tune collision energy function to identify the best collision energy value for these experiments. The standard electrospray ion (ESI) source was used to generate the ions. The concentration of the samples was 10 μg/mL and all samples were injected using a constant flow rate (180 μL/h) of sample solution. The solvent was a chloroform/acetonitrile (10/90, v/v%) mixture. There was no salt or acid addition prior to analysis. A total of 60 scans/spectrum were averaged and the quoted *m*/*z* values are monoisotopic. The ESI-Q-TOF-MS instrument was calibrated in the *m*/*z* range 50–3000 using an internal calibration standard (Tunemix solution) which is supplied from Agilent. All data were processed *via* Bruker Data Analysis software version 4.0.

2.4.2. APCI-Q-TOF-MS and MS/MS measurements

APCI-Q-TOF-MS measurements were performed with a micrOTOF Q-II (Bruker Daltonics) mass spectrometer equipped with an APCI source. APCI-MS was operated with the corona discharge current set to 4000 nA and the capillary voltage was set to 4000 V. Nitrogen was used as nebulizer and drying (desolvation) gas. The vaporizer temperature was set to 400 °C, the drying (desolvation) gas to 250 °C and 4 L/min, and the nebulizer pressure was 2.0 bar. Agilent 1200 Series LC system was utilized to dilute the polymer solutions coming from the syringe pump with the help of a T-fitting. The T-fitting was used to mix the polymer solutions [in chloroform/acetonitrile (10/90, v/v%, 0.5 mg/mL)] from the syringe pump (360 μL/min) and the acetonitrile/water solvent mixture (50/50, v/v%, 0.4 mL/min) from the LC system before the MS interface. Tandem mass (MS/MS) experiments were conducted by isolating the precursor ion of interest in the quadrupole and subjected to collision-induced dissociation (CID).

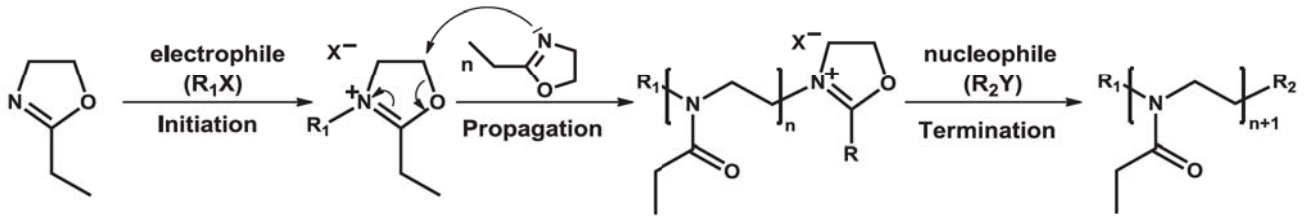
2.4.3. MALDI-TOF-MS and MS/MS measurements

MALDI-TOF-MS experiments were performed with an Ultraflex III TOF/TOF (Bruker Daltonics, Bremen, Germany) equipped with a Nd:YAG laser (smartbeam, 200 Hz) and a collision cell. All spectra were measured in the positive reflector mode. For the MS/MS measurements (LIFT™ mode), argon was used as collision gas at a pressure of 2×10^{-6} mbar and the collision energy amounts to 20 keV. The instrument was calibrated prior to each measurement with an external poly(methyl methacrylate) (PMMA) standard (*m*/*z* 410 or 2500) from PSS Polymer Standards Services GmbH (Mainz, Germany) in the required measurement range. MS and MS/MS data were processed using Flex Analysis 3.0, PolyTools 1.12 (beta version), Data Explorer 4.0 and an isotope pattern calculator. The ion abundances of several scans were summed up to obtain spectra with good signal/noise ratio for MS and MS/MS experiments. The quoted *m*/*z* values are monoisotopic. For the MALDI sample preparation, separate solutions of polymer (10 mg/mL in chloroform), DCTB (30 mg/mL in chloroform), and the doping salt NaI (100 mg/mL in acetone) were prepared and subsequently mixed according to the dried-droplet spotting technique. For each sample 1 μL of the mixture was spotted on a target plate. Each sample was spotted several times and allowed to air-dry at ambient conditions. The laser fired 250 shots per sample spot accumulated from the firing positions (1500 total laser shots per sample).

3. Results and discussion

3.1. Molar mass determination of poly(2-ethyl-2-oxazoline)s

In this study, the various PEtOxs with different end-groups were investigated in detail using ESI, APCI and MALDI MS techniques in a comparative manner. These polymers were synthesized *via* microwave-assisted living CROP and were characterized by SEC to reveal M_n, M_w, and PDI values in order to investigate and prove the synthesis of well-defined polymer systems. As depicted in Scheme 1, functional electrophiles can be applied in order to introduce various initiating (α) end-groups, whereas the terminating (ω) chain ends can be functionalized by attack



Scheme 1. Schematic representation of the cationic ring-opening polymerization of poly(2-oxazoline)s.

Table 1
Characterization results of the investigated PEtOxs.

Compounds	Electrophile	Nucleophile	R_1	R_2
P1	*p*-Toluene-sulfonic acid	Water	H	OH
P2	Methyl *p*-toluene-sulfonate	Water	CH_3	OH
P3	Propargyl*p*-toluene-sulfonate	Water	$CHCCH_2$	OH
P4	Allyl*p*-toluene-sulfonate	Water	CH_2CHCH_2	OH
P5	9-(Chloromethyl)-anthracene	Water	$AnCH_2$	OH
P6	Methyl *p*-toluene-sulfonate	Anthracene-9-carboxylic acid	CH_3	AnCOO
P7	Methyl *p*-toluene-sulfonate	*t*-Butyl 1-piperazine-carboxylate	CH_3	Piptbut
P8	Methyl *p*-toluene-sulfonate	Sodium azide	CH_3	N_3

Table 2
Molar masses and PDI values of the investigated PEtOxs *via* different methods.

Compounds	DP (NMR)	M_n (NMR) (g mol^{-1})	M_n (SEC) (g mol^{-1})	PDI (SEC)	M_n (ESI) (g mol^{-1})	PDI (ESI)	M_n (APCI) (g mol^{-1})	PDI (APCI)	M_n (MALDI) (g mol^{-1})	PDI (MALDI)
P1	12	1.210	1.650	1.25	1.270	1.12	830	1.07	1.475	1.23
P2	19	1.910	2.200	1.1	2.050	1.06	1.060	1.08	2.095	1.05
P3	20	2.040	2.600	1.08	1.940	1.09	1.055	1.05	2.455	1.06
P4	20	2.040	2.600	1.07	1.965	1.06	1.075	1.12	1.935	1.08
P5	18	1.990	2.500	1.11	2.170	1.06	1.020	1.07	2.020	1.06
P6	10	1.230	1.600	1.11	1.230	1.05	960	1.05	1.305	1.05
P7	21	2.285	2.700	1.07	2.295	1.02	995	1.14	2.205	1.03
P8	22	2.240	2.600	1.11	2.430	1.04	870	1.09	2.475	1.03

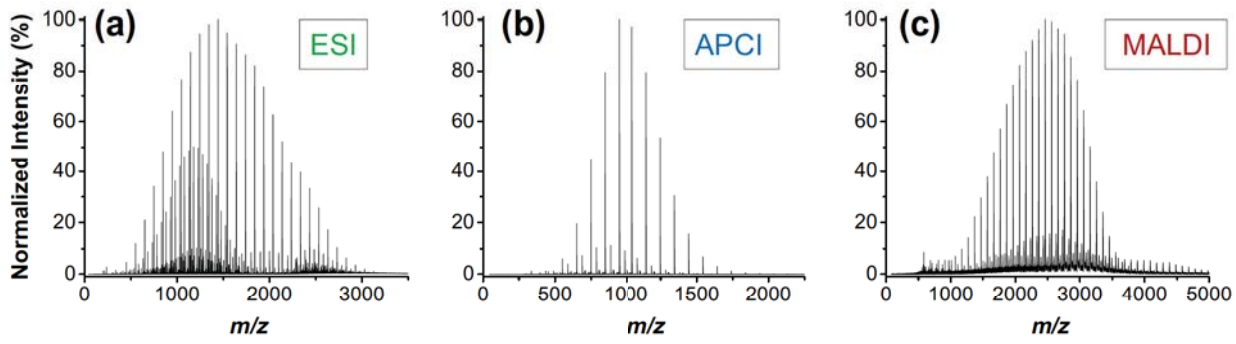


Fig. 1. ESI MS (a), APCI MS (b) and MALDI MS (c) spectra of a representative poly(2-ethyl-2-oxazoline) (P3).

of suitable nucleophiles on the living polymeric oxazolinium species. The compounds used for this purpose are summarized in Table 1.

A comparison of the molar masses and PDI values of the polymers obtained by all applied techniques is provided in Table 2 and ESI, APCI and MALDI mass spectra of the representative PEtOx (**P3**) are presented in Fig. 1. SEC and NMR are favorable and well performed techniques on calculating the molar mass averages of polymers; however, naive extrapolation of the results of these techniques to the case of polymers exhibiting a PDI above 1.1 might lead to significant deviations. As a matter of fact, the molar mass can be miscalculated by SEC as it represents a relative method. Different standards (*e.g.* polystyrene) have to be used for poly(2-oxazoline)s because appropriate SEC calibration standards are missing for these new polymers. As

a consequence, the slightly different molar masses obtained *via* SEC compared to the theoretical molar masses are most likely due to these standards. For an absolute determination of the molar masses of the resulting homopolymers mass spectrometry studies were performed (Table 2). As it can be easily seen from the obtained results, the APCI technique does not seem to be an adequate tool for the molar mass determination. The molar mass distributions in MALDI and ESI MS measurements are more representative of the actual molar mass distributions. However, because of the highest tendency of all three techniques to favor the ionization of lower molar mass species (the mass discrimination effect), APCI MS should not be considered to provide the true molar mass distributions of PEtOxs. On the other hand, APCI allows the detection on a wide range of polarity of sample/solvent and seems to be complementary to ESI and MALDI. Moreover, the coupling of APCI instruments to various LC systems is possible (SEC, reversed-phase and normal-phase LC). This combination enables the investigation of synthetic polymers up to certain molar mass values [27]. Therefore, the investigation of synthetic polymers with APCI and complementary tandem mass experiments to elucidate fragmentation processes will provide crucial knowledge for future LC-APCI MS and MS/MS studies. However, APCI-MS represents a less suited technique to obtain molar mass information from larger macromolecules and it should be used as a complementary technique to ESI and/or MALDI.

ESI MS provided multiply charged species (+2 and +3) in the lower mass region, whereas, APCI and MALDI provided only singly charged species due to their ionization process (only singly charged species are the lucky survivors in these techniques) [28]. Nonetheless, ESI MS measurements provide perfectly suited data for the reconstruction of the polymer structure and composition. Multiply charged species do not overlap with the singly charged peaks, which enable a straightforward interpretation. Moreover, it is possible to deconvolute ESI MS data to obtain singly charged spectra for a correct molar mass calculation, thus the existence of multiply charged species does not represent any difficulties (Figs. S1 and S2, see Supporting Information (SI)). These observations were valid for all investigated PEtOx samples (**P1**–**P8**). However, one should be always careful about the values obtained from the deconvolution of ESI MS data since there is no currently available deconvolution technique which takes into account the entire complex physical dependencies of the charge envelopes observed in MS on the true molar mass distributions of macromolecules.

3.2. End-group determination of poly(2-ethyl-2-oxazoline)s

The main aim of this study was to analyze PEtOxs with different end-groups *via* three soft ionization methods (ESI, APCI and MALDI) in a systematic manner to decide which method is best suited for the characterization of these polymers. Next to the molar mass determination, all three methods were applied to analyze these polymers in order to obtain compositional information *i.e.* the determination of end-groups which can be obtained by single-dimensional mass spectrometry (MS). One representative PEtOx (**P3**) is selected to explain the details of the MS analysis and to discuss the difficulties related to isomeric side products. **P3** was initiated with propargyl-*p*-toluenesulfonate and terminated with water. As reported earlier two different side reactions may occur during the polymerization process. On the one hand proton initiated polymer chains can be formed by a chain transfer reaction. On the other hand, water can attack the oxazolinium species at two different positions causing two different end groups, namely a hydroxyl end-group [$C_3H_3(C_5H_9NO)_nOH$] or an amine/ester end-group [$C_3H_3(C_5H_9NO)_{n-1}NHC_2H_4CO_2C_2H_5$]. However, the latter have identical *m*/*z* values and it is not possible to differentiate between them *via* single-stage mass spectrometry experiments. Nevertheless, the existence of the side product (with an amine/ester end-group) can be confirmed *via* tandem MS experiments due to the different fragmentation patterns, which makes their application obligatory in order to characterize individual end-groups and to identify macromolecular sequences and architectures.

Details of the ESI, APCI and MALDI mass spectra of the representative PEtOx (**P3**) are presented in Fig. 2. With all three MS techniques it was possible to analyze the polymer sample. The main distribution represents the desired polymer structure with the expected end-groups [R_1–$(C_5H_9NO)_n$–R_2, R_1: $-C_3H_3$, R_2: –OH]. The less intense signals can be assigned to proton initiated polymer chains [R_1: –H, R_2: –OH]. (Table 3) The spacing between the main

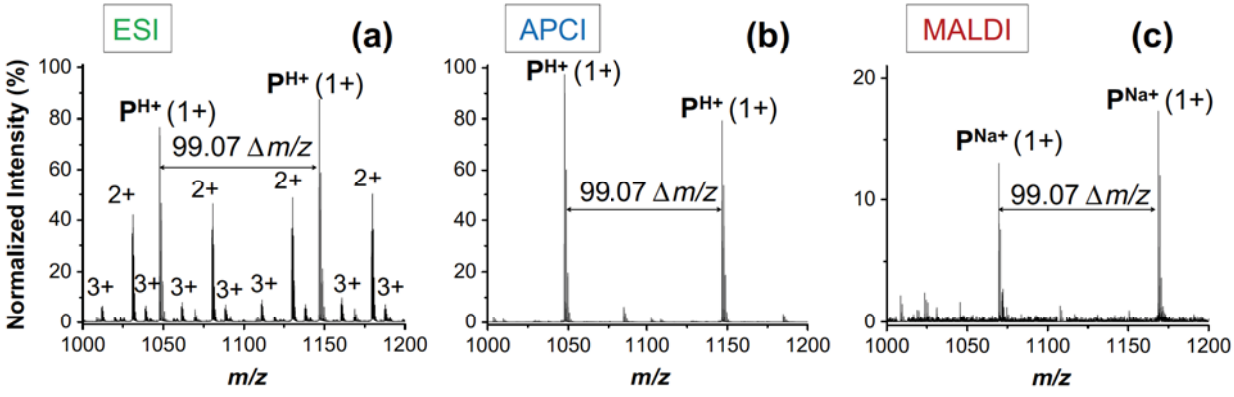


Fig. 2. ESI MS (a), APCI MS (b) and MALDI MS (c) spectra of a representative poly(2-ethyl-2-oxazoline) (**P3**) (zoom into the area between 1000 and 1200*m*/*z*).

distribution peaks are in accordance to one repeating unit of PEtOx (99.07 $\Delta m/z$). ESI and APCI provided protonated species ($\mathbf{P^{H+}}$) and MALDI delivered sodiated species ($\mathbf{P^{Na+}}$) in their MS spectra. This situation was expected, because the ESI and APCI sample preparations were performed in pure solvents whereas the MALDI sample preparation includes the combination of DCTB matrix and NaI salt to improve ionization in MALDI process. Overall, it can be observed that ESI and APCI allow a greater number of end-groups to be detected when compared to MALDI for most of the PEtOx polymers (**P1–P8**). The detailed peak assignments of the ESI, APCI, and MALDI MS spectra of PEtOx (**P3**) are provided in Table 3.

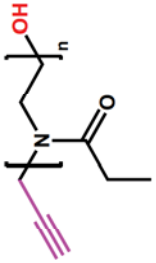
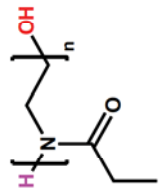
End-group analysis *via* different MS techniques showed that MALDI MS caused some cleavages (partial and/or complete end-group cleavages) during the single-dimensional MS for certain PEtOx samples such as **P6–P8**. For instance, terminal azide group cleavage has occurred *via* in-source fragmentation during the MALDI process for **P8**. This situation was not observed for the ESI and APCI MS analysis. Moreover, ester bond cleavages have been observed in MALDI MS for **P6** and **P7** and a lower quality signal-to-noise ratio (S/N) was observed for MALDI analysis compared to ESI and APCI. It is noteworthy that the sample preparation in MALDI experiments should result in homogeneous matrix and sample co-crystallization to obtain valuable and reproducible data. The traditional dried droplet method used in this study was questionable to be the best way of accomplishing this, because it might create inhomogeneous polymer crystals on MALDI target. Moreover, it represents a more demanding sample preparation procedure than compared to ESI and APCI. Therefore, ESI and APCI should be considered as method of choice if the maximum numbers of end-group functionalities within given polymer samples are to be identified.

Concisely, the performance of the ESI and APCI methods was found to be comparable in respect of end-group determination and both techniques generally resulted in less in-source fragmentation compared to the MALDI technique. Taking everything into account, conclusive remarks from this study for the investigation of PEtOx systems are: (1) ESI and APCI are superior for end-group determination of PEtOxs. (2) ESI MS can be used for molar mass determination up to certain values; however, for high molar mass polymers multiply charged species might cause problems. This was not the case for the present study, because all polymers were intentionally synthesized with lower molar masses (1500–2500 g/mol). (3) APCI is not suitable for the molar mass determination of samples with molar masses higher than 1500 g/mol. However, it is the best technique to be coupled with different LC techniques; therefore, APCI MS studies of synthetic polymers are of significant interest and need to be addressed. (4) MALDI is suitable for the molar mass determination, but not the best method for end-group analysis.

3.3. Tandem mass spectrometry of poly(2-ethyl-2-oxazoline)s

Single-dimensional mass spectrometry (MS) can identify the number and types of monomer units, backbone substituents, and end-groups of the studied polymer.

Table 3
Detailed peak assignments of the ESI, APCI, and MALDI MS spectra of PEtOx (**P3**).

Compounds	Structure	Theoretical m/z	m/z (ESI)	m/z (APCI)	m/z (MALDI)
Desired product $C_3H_3(C_5H_9NO)_nOH$		1047.7176 (H^+) 1069.6995 (Na^+)	1047.7131	1047.7115	1069.693
Side product 1 $C_3H_3(C_5H_9NO)_nO_2C_5H_{10}N$ (with an ester end-group obtained during the termination process)		1047.7176 (H^+) 1069.6995 (Na^+)	1047.7131	1047.7115	1069.693
Side product 2 $H(C_5H_9NO)_nOH$ (proton initiated polymer due to the chain transfer reactions)		1009.7020 (H^+) 1031.6839 (Na^+)	1009.6929	1009.7045	1031.702

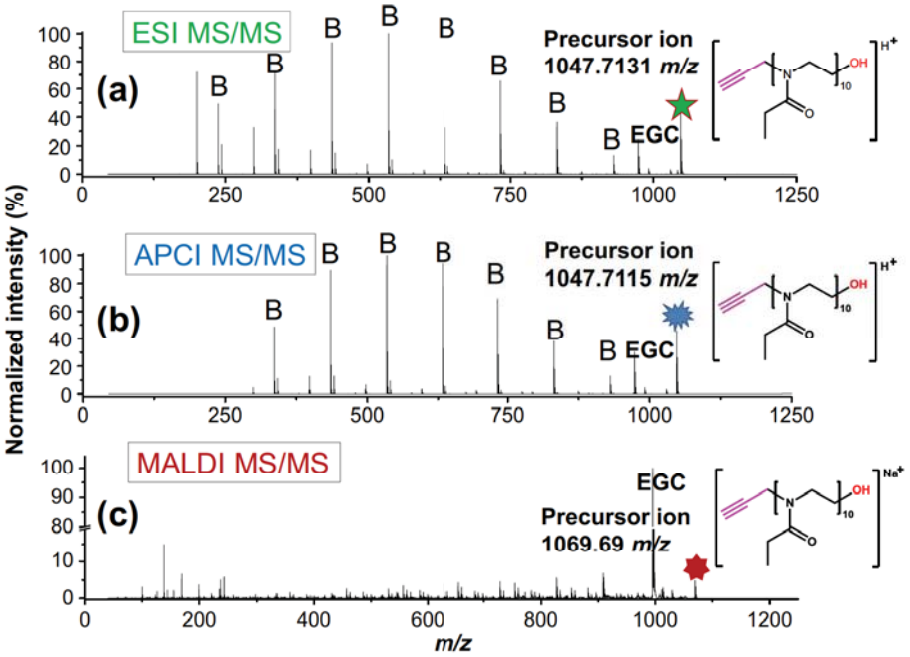


Fig. 3. ESI (a), APCI (b) and MALDI (c) tandem mass spectra of a representative poly(2-ethyl-2-oxazoline) (**P3**) (EGC: end-group cleavage, B: fragmentation products from depolymerization process).

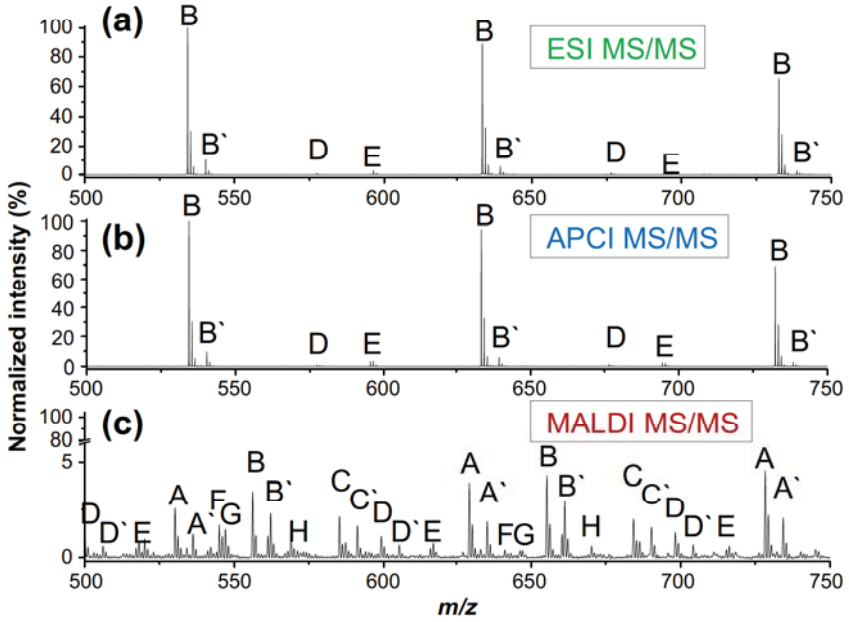
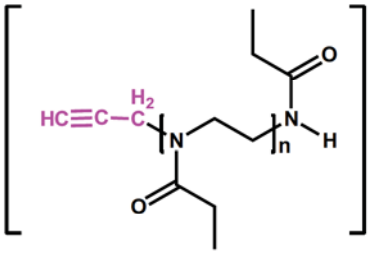
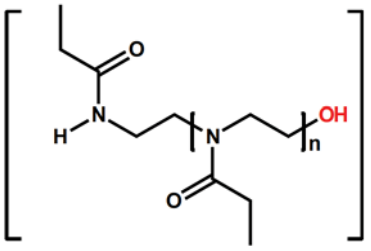
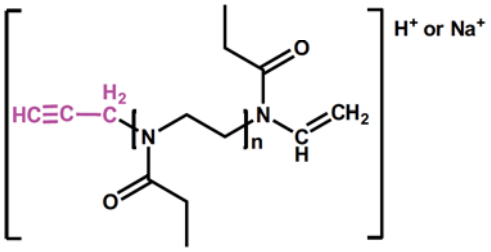
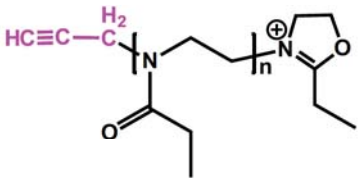
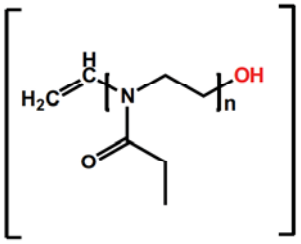
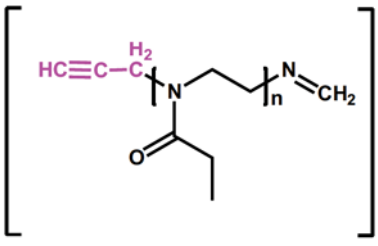


Fig. 4. ESI (a), APCI (b) and MALDI (c) tandem mass spectra of a representative poly(2-ethyl-2-oxazoline) (**P3**) zoom into the area between 500 and 750*m*/*z*.

However, molar mass information alone is not sufficient to distinguish isobaric and isomeric species to determine macromolecular connectivities and architectures completely. For this purpose, a detailed end-group characterization of synthetic polymers requires tandem mass spectrometry (MS/MS) experiments to propose the possible fragmentation pathways to draw conclusions about the main structure. The obtained diagnostic fragmentation products assist the elucidation of the detailed molecular composition. Therefore, in depth tandem mass spectrometry measurements were performed to evaluate these methods (ESI, APCI and MALDI) as structural characterization tools for the detailed analysis of the PEtOxs samples. Eight different PEtOxs with different initiating (α) and terminating (ω) end-groups were investigated in order to elucidate the effect of end-groups on the fragmentation mechanism (**P1**–**P8**).

ESI, APCI and MALDI tandem mass spectra of a representative PEtOx (**P3**) are displayed in Fig. 3. For the tandem MS investigations, all ions with good signal intensities within the main distributions were selected as precursor ions in order to investigate the fragmentation mechanisms.

Table 4
Detailed peak assignments of the fragmentation products of PEtOx (**P3**) obtained *via* ESI, APCI, and MALDI MS/MS spectra.

Fragments	Structures	Chemical formula – theoretical *m/z*	*m/z* (ESI)	*m/z* (APCI)	*m/z* (MALDI)
A		$C_3H_3(C_5H_9NO)_nNC_3H_6O$ 607.4177 (H^+) 629.3997 (Na^+)	n.a.	n.a.	629.285
A′		$C_5H_{10}NO(C_5H_9NO)_nOH$ 613.4283 (H^+) 635.4103 (Na^+)	n.a.	n.a.	635.273
B	 or 	$C_3H_3(C_5H_9NO)_nNC_5H_9O$ 633.4334 (H^+) 655.4153 (Na^+)	633.4269	633.4286	655.244
B′		$C_2H_3(C_5H_9NO)_nOH$ 540.3755 (H+) 562.3575 (Na+)	540.3692	540.3692	562.200
C		$C_3H_3(C_5H_9NO)_nNCH_2$ 563.3915 (H^+) 585.3735 (Na^+)	n.a.	n.a.	585.204

(*continued on next page*)

Table 4 (*continued*)

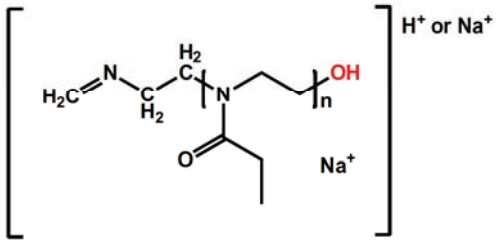
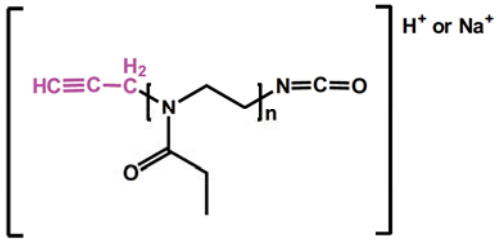
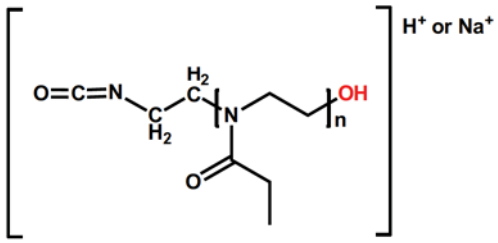
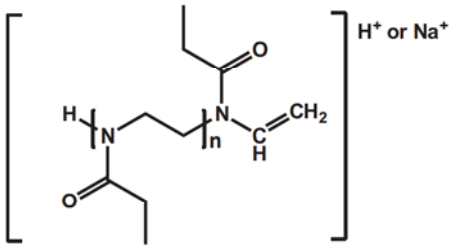
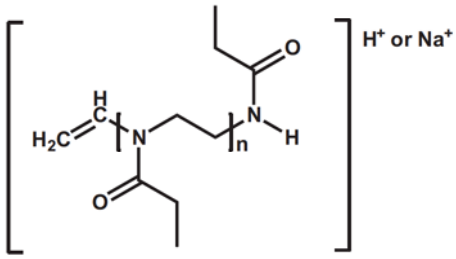
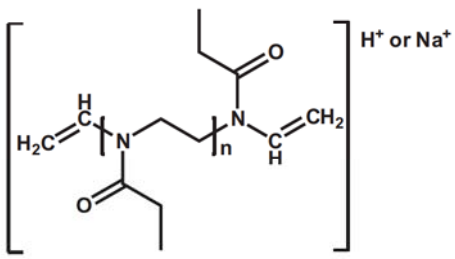
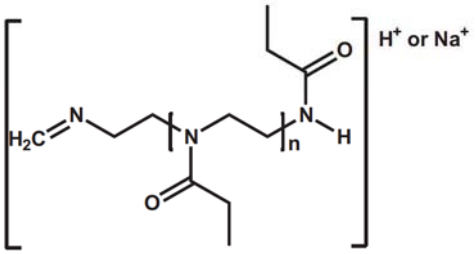
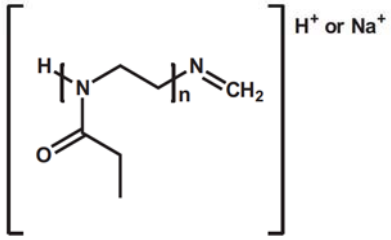
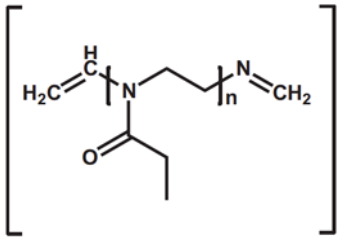
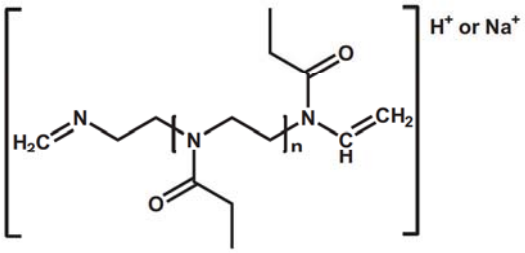
Fragments	Structures	Chemical formula – theoretical *m*/*z*	*m*/*z* (ESI)	*m*/*z* (APCI)	*m*/*z* (MALDI)
C′		$C_3H_6N(C_5H_9NO)_nOH$ 569.4021 (H^+) 591.3841 (Na^+)	n.a.	n.a.	591.190
D		$C_3H_3(C_5H_9NO)_nNCO$ 577.3708 (H^+) 599.3527 (Na^+)	577.4028	577.4024	599.171
D′		$C_3H_4NO(C_5H_9NO)_nOH$ 583.3814 (H^+) 605.3633 (Na^+)	583.4133	583.4171	605.171
E	 or 	$H(C_5H_9NO)_nNC_5H_9O$ or $C_2H_3(C_5H_9NO)_nNC_3H_6O$ 595.4177 (H^+) 617.3997 (Na^+)	595.4168	595.4159	617.255
F		$C_2H_3(C_5H_9NO)_nNC_5H_9O$ 622.4412 (H^+) 644.4232 (Na^+)	n.a.	n.a.	644.117

Table 4 (*continued*)

Fragments	Structures	Chemical formula – theoretical *m/z*	*m/z* (ESI)	*m/z* (APCI)	*m/z* (MALDI)
G	 or 	$C_3H_6N(C_5H_9NO)_nNC_3H_6O$ or $H(C_5H_9NO)_nNCH_2$ 624.4443 (H^+) 646.4262 (Na^+)	n.a.	n.a.	646.273
H	 or 	$C_2H_3(C_5H_9NO)_nNCH_2$ or $C_3H_6N(C_5H_9NO)_nNC_5H_9$ 650.4599 (H^+) 672.4419 (Na^+)	n.a.	n.a.	672.259

ESI and APCI MS/MS provided the same fragmentation products, whereas MALDI MS/MS delivered a large number of additional fragmentation products. Tandem MS analysis of the PEtOxs *via* MALDI MS/MS revealed the elimination of small molecules such as ethene and hydrogen in their fragmentation patterns. Additionally, a McLafferty rearrangement can be a possible fragmentation route for these polymers [24]. However, the main fragmentation mechanism observed from the ESI and APCI MS/MS studies was the depolymerization mechanism (can also be called as unzipping mechanism or monomer evaporation) [25]. One reason for this observation is most likely the different cationization agent. The precursor ions were protonated in ESI and APCI analysis ($\mathbf{P^{H+}}$:1047.7131*m/z* for ESI and 1047.7115*m/z* for APCI), but sodiated in MALDI analysis ($\mathbf{P^{Na+}}$: 1069.693*m/z*). The cationization agent can have a crucial influence on fragmentation mechanism. Another reason for the differences between the tandem MS techniques is probably the selective ionization in the employed ESI and APCI processes. This situation can mainly be attributed to a favorable ionization of lower abundance species (isomeric species in the case of **P3**) when using ESI and APCI MS. The fragmentation of the side product (with an amine/ester end-group $[C_3H_3(C_5H_9NO)_{n-1}O_2C_5H_{10}N]$) is a

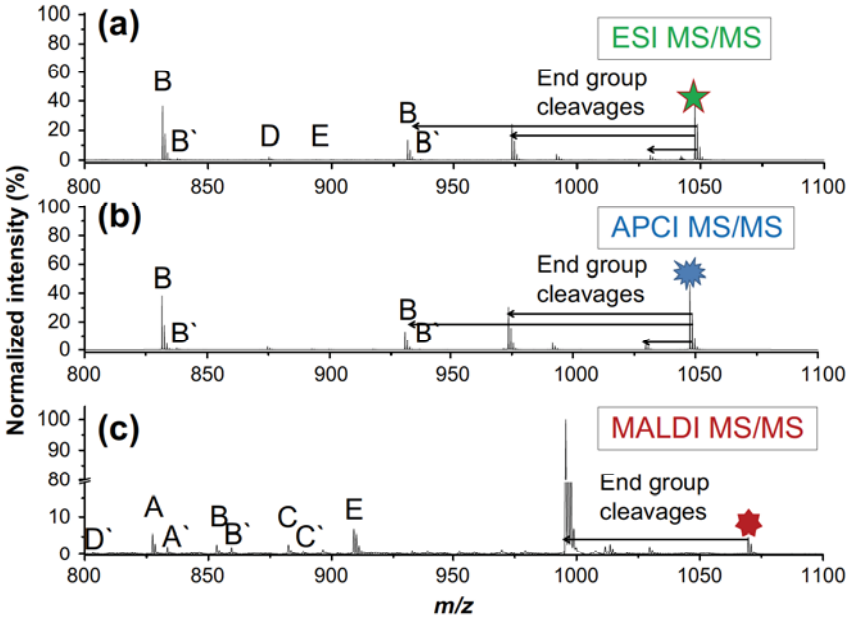
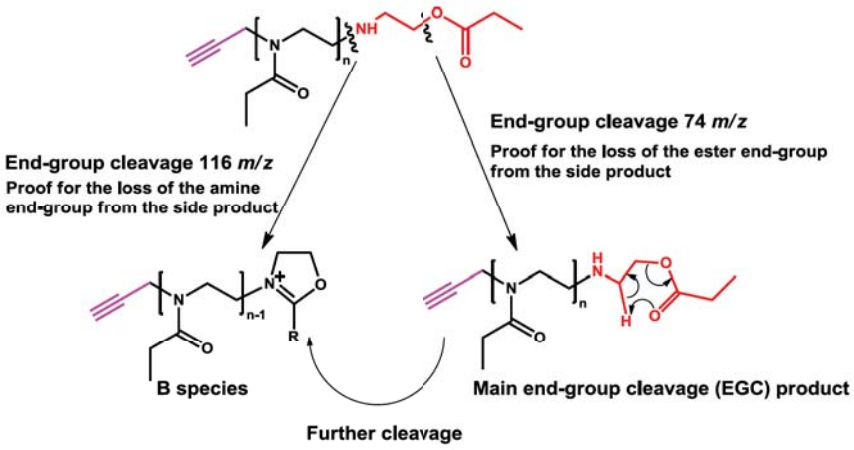


Fig. 5. ESI-Q-TOF (a), APCI-Q-TOF (b) and MALDI-TOF (c) tandem mass spectra of a representative poly(2-ethyl-2-oxazoline) (**P3**), upper mass region.

lot easier than the fragmentation of the main product (with a hydroxyl end-group [$C_3H_3(C_5H_9NO)_nOH$]), which requires higher energies to cleave the bond between the polymer backbone and end-group functionality. This observation was valid for all PEtOx polymers with hydroxyl end-group functionality. Therefore, a favorable ionization of lower abundance isomeric species in ESI and APCI can provide an explanation for the different fragmentation products obtained *via* different ionization techniques. The tandem mass analyses were performed at various precursor ions (with different molar masses, *e.g.*, PEtOxs with 5, 10, 20 and 25 monomer units) to observe the effect of the molar mass on the fragmentation pathways. The comparison between the different precursor ions shows the same fragmentation series. No differences for different precursor ions for the studied PEtOxs could be observed (Fig. S3, see Supporting Information (SI)).

The details of tandem mass spectra (ESI, APCI and MALDI MS/MS) of **P3** are displayed in Fig. 4 (zoom into area between 500 and 750m/z). The comprehensive analysis of the diagnostic fragmentation products obtained from CID experiments provided the information necessary to identify these products and how they were formed (Table 4). Firstly, the protonated species (in ESI and APCI) enabled partial or complete end-group cleavages (*e.g.* of water (H_2O)) from the desired product and ester as well as amine cleavages ($O_2C_3H_6$ and $-NHC_2H_4CO_2C_2H_5$) from the side product (in the upper mass region). This situation confirmed the existence of the side product with the same m/z value (Fig. 5). The cleavage of the ester end group by 1,5-hydrogen rearrangement and the amine cleavage from the side product are depicted in Scheme 2. However, the intensities of fragmentation product ions cannot represent their amount in the polymer mixture. On the one hand, a number of end-groups can be cleaved off easily without so much trouble (*e.g.* ester end-group in the side product), on the other hand some end-groups cannot be fragmented even though higher collision energies are utilized in the



Scheme 2. Schematic representation of the cleavages of the ester and the amine end groups from the side product.

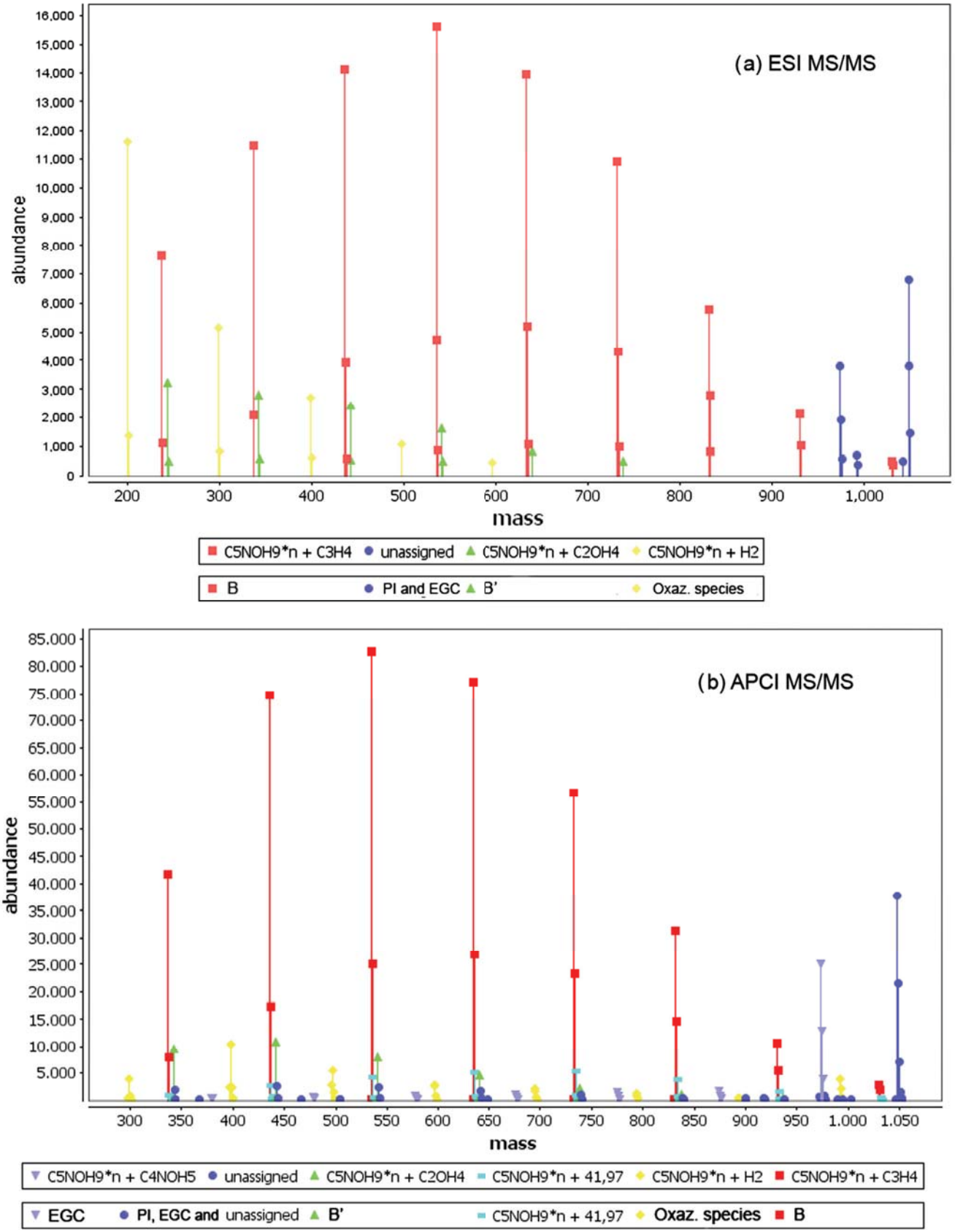


Fig. 6. ESI and APCI MS/MS spectra of **P3**: evaluation by the PLUMS software (EGC: end-group cleavage, PI: precursor ion).

CID experiments (*e.g.* –OH end-group in the main product). Therefore, it is not possible to decide which product (desired or side product) is present in higher amounts in the polymer sample; the high intensity of ester end-group

cleavage fragmentation product in tandem MS data is not a proof that this side product is present in higher amounts (the signal intensity in tandem MS analysis is not quantitative). Subsequent to the end-group cleavages, depolymerization occurred to form **B** species (the main fragmentation product ion series in ESI and APCI, see Scheme S1 Supporting Information (SI)). In contrast, MALDI tandem MS data revealed the fragmentation of sodiated species *via* the elimination of ethene and hydrogen from the backbone to form **A**, **A′**, **B** and **B′** species. Those fragmentation products can also be formed *via* a McLafferty rearrangement mechanism as already explained in a previous study (Schemes S2 and S3 (SI)) [25]. **C** and **C′** fragmentation product ion series could be explained by an α-cleavage of the backbone between the CH_2 groups followed by an elimination of side groups on the PEtOx backbone. **D** and **D′** fragment ion can be formed because of the ethylene elimination of the side groups. Finally, the less intense **E**, **F**, **G**, and **H** series can be obtained from the combination of four possible fragmentation mechanisms mentioned above [24]. These observations gained *via* different tandem MS techniques for **P3** are valid for all investigated PEtOx samples (**P1**–**P8**).

3.4. Application of PLUMS software for the interpretation of tandem MS data

Finally, the PLUMS software was utilized to identify fragmentation products in an automated manner [29]. Interpretation of MALDI data of PEtOx by this software was already shown in a previous study [30]. However, the application of this data interpretation software to ESI and APCI tandem MS data is reported for the first time in this contribution. The manual interpretation of tandem mass spectra of synthetic polymers is laborious and time-consuming. Therefore, the application of this software tool accelerates the interpretation of tandem mass spectra obtained without requiring any further knowledge about the polymer class or the fragmentation behavior. The PLUMS software only requires a peak list of the measured polymer sample as a XML file for the evaluation of the tandem mass spectrum. Fig. 6 represents the evaluation of ESI and APCI MS/MS spectra of **P3**. The software provided a value for the repeating unit of *m*/*z* 99.07 within different fragmentation series. The calculated value *m*/*z* 99.07 fits exactly to the chemical formula of the EtOx repeating unit (C_5H_9NO). The automatic interpretation delivered several fragmentation series, which are ascribed to the depolymerization mechanism which were already assigned manually. Defining the characteristic fragmentation patterns for a certain type of synthetic polymer is important and this information can be used as a reference to characterize unidentified polymers within the same class. To accomplish this purpose, the PLUMS software enables polymer scientists to achieve straightforward identification of fragmentation patterns for synthetic polymers without laborious manual interpretation efforts.

In conclusion, the knowledge gained from this contribution will also support to construct a tandem MS product ion library of POxs with different end-groups including fragmentation pathways which will offer important information for the future to enable a fast and computerized identification of these polymer. Moreover, the software studies will allow the characterization of synthetic polymers by tandem MS in a new way (Polymeromics) [31], similar as in the fields of proteomics, metabolomics, genomics and glycomics.

4. Conclusions

In this contribution, three soft ionization methods (ESI, APCI and MALDI) were compared for the first time for the detailed analysis of PEtOxs. Eight PEtOx polymers with different end-groups were investigated using the ESI, APCI and MALDI MS and MS/MS techniques in terms of molar mass and molecular composition. ESI and APCI methods proved to be capable of detecting all end-groups attached to PEtOxs and they are even more useful when MS/MS techniques are applied. However, the MALDI MS technique is superior to obtain molar mass information about these polymers. End-group analysis of these polymers *via* MS/MS clearly indicated that the depolymerization mechanism was the predominant occurring fragmentation mechanism in ESI and APCI MS/MS studies. But also, to a lower extent, the elimination of small molecules such as ethene and hydrogen can be observed in their fragmentation patterns and a McLafferty rearrangement is a possible fragmentation route for these polymers. These mechanisms (all backbone and side group cleavages) were the main fragmentation mechanisms in MALDI-TOF MS/MS analysis. The multiple fragmentation pathways that complicated the MALDI MS/MS analyses are almost completely suppressed in ESI and APCI MS/MS. This fact is making the interpretation of spectra easier and may help to introduce end-group analysis *via* MS/MS techniques to non-MS-specialists. Moreover, the successful application of a special software for the interpretation of tandem MS data of polymers was shown. In summary, complementary information obtained from these tandem MS experiments will be valuable for the analysis of PEtOxs in the future. The insights gained from this work will be a part of the recently introduced research field "Polymeromics" and the obtained information will assist the structural elucidation of various classes of synthetic polymers.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eurpolymj.2013.02.008.

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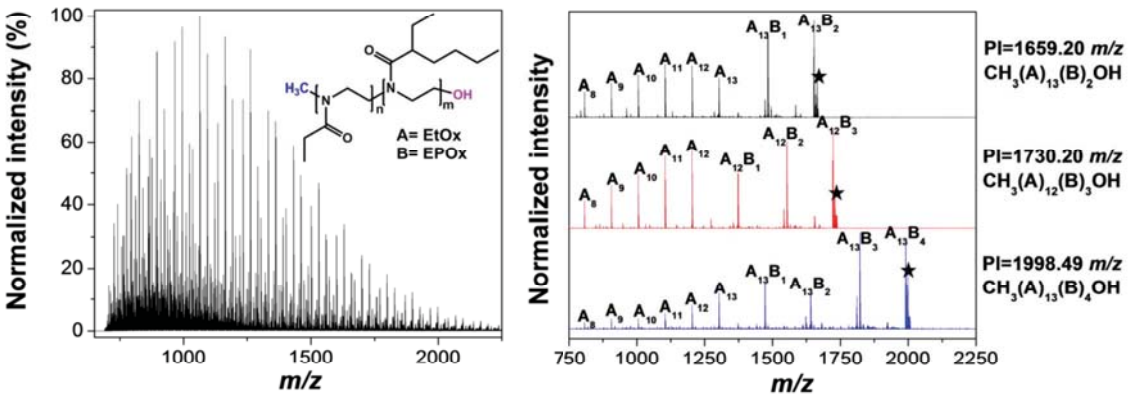
Publication P8:

Characterization of different poly(2-oxazoline) block copolymers by MALDI-TOF MS/MS and ESI-Q-TOF MS/MS

Anja Baumgaertel, Esra Altuntaş, Kristian Kempe, Anna Crecelius, and Ulrich S. Schubert

Journal of Polymer Science, Part A: Polymer Chemistry **2010**, *48,* 5533-5540.



Characterization of Different Poly(2-oxazoline) Block Copolymers by MALDI-TOF MS/MS and ESI-Q-TOF MS/MS

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ABSTRACT: A complete library of poly(2-oxazoline) block copolymers was synthesized via cationic ring opening polymerization for the characterization by two different soft ionization techniques, namely matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF MS). In addition, a detailed characterization was performed by tandem MS to gain more structural information about the block copolymer composition and its fragmentation behavior. The fragmentation of the poly(2-oxazoline) block copolymers revealed the desired polymer structure and possible side reactions, which could be explained by different mechanisms, like 1,4-ethylene or hydrogen elimination and the McLafferty +1 rearrangement. Polymers with aryl side groups showed less fragmentation due to their higher stability compared to polymers with alkyl side groups. These insights represent a further step toward the construction of a library with fragments and their fragmentation pathways for synthetic polymers, following the successful examples in proteomics. © 2010 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 48: 5533–5540, 2010

KEYWORDS: block copolymers; ESI; MALDI; poly(2-oxazoline); mass spectrometry

INTRODUCTION Poly(2-oxazoline)s are an important class of synthetic polymers in different application fields, like for thermosensitive materials, for sensors, or for active agents in drug delivery as carrier systems.[1–4] The cationic ring opening polymerization (CROP) of 2-oxazolines was first reported in the 1960s[5–8] and the living polymerization mechanism was also confirmed by MS.[9] Because of the versatility of 2-oxazoline monomers, the synthesis of a library of well-defined copolymers with special properties, like self-organizing features as well as thermoresponsive behavior[10–13] is possible, as a consequence, they represent ideal candidates for the concept of functional multicompartment micelles.[1,14–16]

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF MS) are used as a common characterization method for synthetic polymers in the last years.[17–20] Both techniques belong to the soft ionization methods in MS besides fast atom bombardment or secondary ion MS to analyze proteins, peptides, as well as synthetic polymers.[21] The main difference between the first two ionization techniques is the m/z range that differs from more than 100,000 g/mol (MALDI) to less than 4,000 g/mol (ESI).[22] On the other hand, the characterization by ESI-Q-TOF MS requires only less concentrated solutions and no extensive search for the right sample preparation method and matrix/salt combination compared with MALDI-TOF MS. ESI-Q-TOF MS only requires the selection of the best suited solvent to dissolve completely the polymer. Additionally, ESI-Q-TOF MS seems to be the "softer" ionization process because also less stable polymers could be measured by this technique.[22] Tandem MS allows a further analysis by determining possible fragmentation pathways of selected ions by a collision-induced dissociation (CID) cell. This MS/MS technique elucidates additional structural information about the polymer itself and its polymerization reaction and has been already used for the characterization of several synthetic homopolymers and block or random copolymers, like poly(methyl methacrylate) (PMMA), poly(ethylene glycol) (PEG), poly(2-ethyl-2-oxazoline) p(EtOx), or poly(ethylene glycol)-*b*-poly(styrene) (PEG-*b*-PS).[23–26]

In the current contribution, poly(2-oxazoline) block copolymers were synthesized to study the behavior of the polymers using different MS techniques. Additionally, the fragmentation behavior of the different side groups, like aryl or alkyl, was investigated in detail. Here, we present the characterization of some block copolymers by MALDI-TOF and ESI-Q-TOF MS as well as by tandem MS analysis. This study

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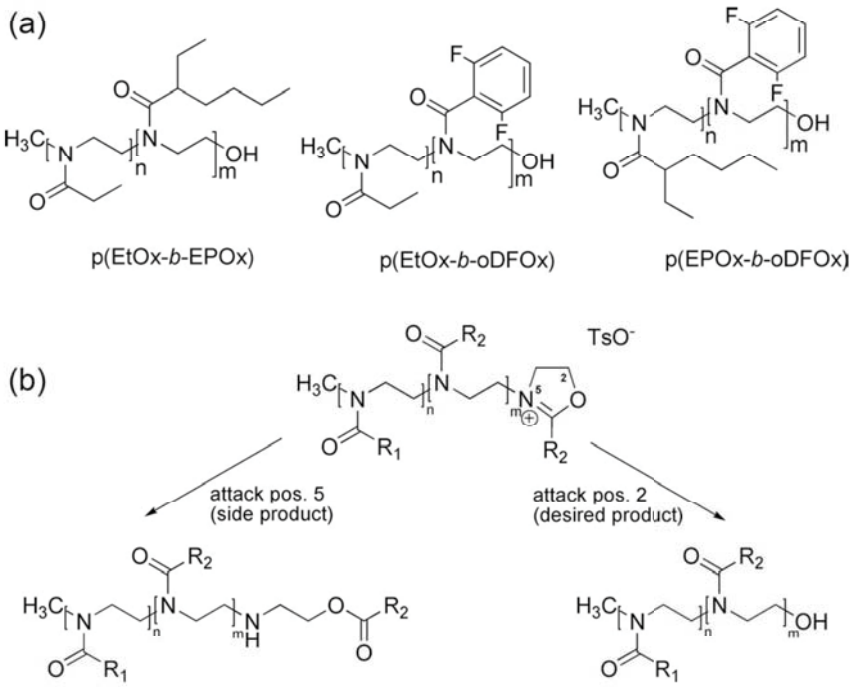


SCHEME 1 (a) Schematic representations of the investigated poly(2-oxazoline) block copolymers. (b) Possible termination reactions with water as end-capping agent.

confirms the block copolymer structure and illustrates the fragmentation behavior within the polymer chain.

RESULTS AND DISCUSSION

The synthesis of different poly(2-oxazoline) block copolymers can be performed via a CROP. By means of microwave-assisted heating,[27–29] the block copolymers were synthesized in a living manner by adding the second monomer after a complete conversion of the first monomer. In this study, all block copolymers were initiated with methyl tosylate and end capped with water. The detailed schematic structures of the poly(2-oxazoline) block copolymers, which are discussed in the present study, are depicted in Scheme 1(a). As possible side reaction in the termination step, water can attack not only position 2 but also position 5 yielding an ester end group [see Scheme 1(b)]. This could be confirmed by tandem MS as shown later. The compounds were characterized by size-exclusion chromatography (SEC) to obtain the molar mass (M_n and M_w) and the polydispersity index values of the synthesized block copolymers. The comparison of these values with those that can be achieved from the MALDI-TOF MS measurements by using the Data Explorer resulted in a good agreement between the two characterization methods (see Table 1). For the analysis with MALDI, it is known that mass discrimination effects can occur, which can lead to a shift of the signal maximum to a lower m/z regime of a distribution.[30,31] The M_n and M_w values of the analyzed block copolymers showed this mass discrimination effect of the MALDI-TOF MS results in comparison with those, which were obtained by SEC. The peak maxima were shifted slightly in all distributions (see Table 1).

Additionally, the characterization by MALDI-TOF MS can be used to investigate the occurrence of possible side reactions, like chain transfer or chain coupling, as well as to determine possible end groups. All spectra are measured in the reflector mode to confirm the copolymer structure by comparison of the measured and the theoretical calculated isotopic pattern. The MALDI-TOF MS spectra are displayed in Figure 1. The block copolymer p(EtOx-*b*-EPOx) contains two main distributions A (methyl initiated) and B (proton initiated) consisting of different block length of the two monomers [Fig. 1(a)]. This second distribution B can be explained by chain transfer reactions during the polymerization process. In the zoom of p(EtOx-*b*-EPOx), the varying length of the two blocks is displayed by the number of monomer units of the first and the second monomers, for example, 5–5 (first monomer–second monomer) [see Fig. 1(b)]. In the same way, the other two block copolymers were analyzed in the MS mode [Fig. 1(c,d)]. The existence of a side product (initiated by a proton) could be confirmed in both spectra. Unfortunately, this behavior during the reaction led to overlapping signals of the different distributions. Due to this effect, a small amount of H(oDFOx)$_m$OH homopolymer in the spectrum was observed in the MS spectrum of p(EPOx-*b*-oDFOx), which can be explained by chain transfer reactions. The existence of these two different possible structures could be proven by tandem MS as discussed later. Additionally, homopolymer

TABLE 1 Molar Mass Values of the Investigated Poly(2-oxazoline) Block Copolymers

	M_n (SEC) (g/mol)	PDI (SEC)	M_n (MALDI) (g/mol)	PDI (MALDI)
p(EtOx-*b*-EPOX)	1,800	1.13	1,590	1.04
p(EPOx-*b*-oDFOx)	1,950	1.2	2,060	1.12
p(EtOx-*b*-oDFOx)	2,010	1.15	2,120	1.05

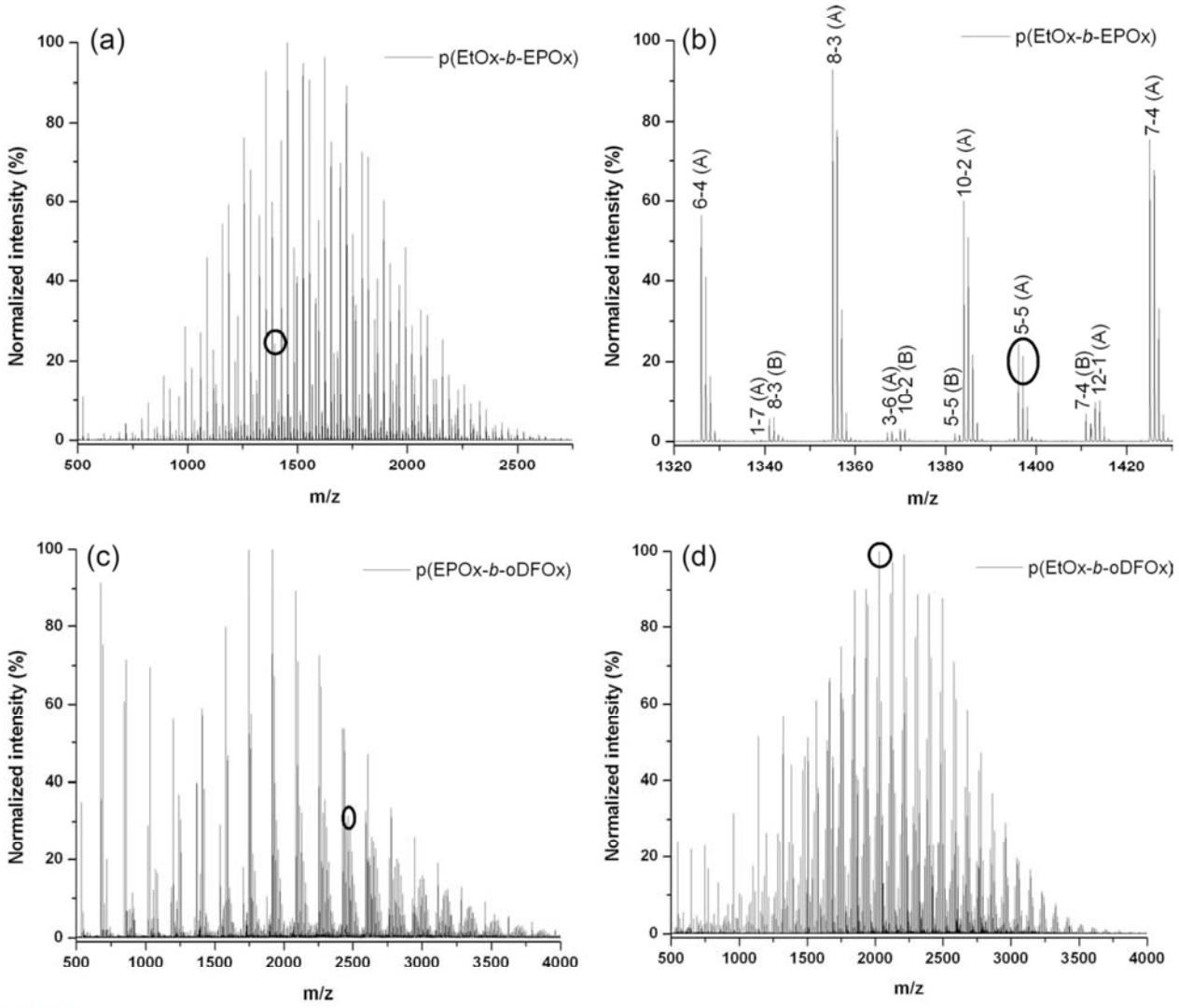


FIGURE 1 MALDI-TOF MS spectra of different poly(2-oxazoline) block copolymers: (a) p(EtOx-*b*-EPOx), (b) zoom into the MS spectrum of p(EtOx-*b*-EPOx) (*m/z* 1320–1430), (c) p(EPOx-*b*-oDFOx), and (d) p(EtOx-*b*-oDFOx).

of EPOx (initiated with methyl and terminated with hydroxyl) could be found in the p(EtOx-*b*-oDFOx) sample, which can be explained by the termination of polymer chains during the polymerization.

For the tandem MS investigations, one precursor ion of each block copolymer MS spectrum (marked with a circle in Fig. 1) was chosen and the obtained spectra are displayed in Figure 2. The MS/MS spectra of all measured poly(2-oxazoline) block copolymers can be divided into three main parts. In the lower molar mass region (up to *m/z* 500), the spectra are crowded and the signals showed very high intensities with the formation of positively charged monomer units of the first building block with methyl as initiating group. This fragmentation series can be explained by main chain scission of the back bone and is marked with an **M** in the MS/MS spectra. The middle part displays a varying number of fragmentation series of the different block copolymers, which are discussed later in detail for p(EtOx-*b*-EPOx), and in the upper molar mass region, one intensive signal next to the parent peak can be observed in all tandem MS spectra. This intensive signal can be assigned to the neutral loss of the ester end group of the second possible parent peak structure. The fragmentation can be explained by a McLafferty +1 rearrangement (1,5 hydrogen transfer) that includes the ester bond leading to the cleavage of the ester and the formation of a $NHCH{=}CH_2$ end group at the copolymer chain as shown in Scheme 2(a).[32,25] Due to that dominant signal in the tandem MS spectrum, the formation of an ester end group during the polymerization as possible side reaction can be confirmed. Based on the results of the p(EtOx) homopolymers, the fragmentation mechanisms were assigned to the different block copolymers.[26] As an example, the fragmentation behavior of p(EtOx-*b*-EPOx) in a CID cell with argon as collision gas is discussed in more detail. Figure 2(a) displays the complete tandem MS spectrum (precursor ion *m/z* 1396) with five repeating units of 2-ethyl-2-oxazoline

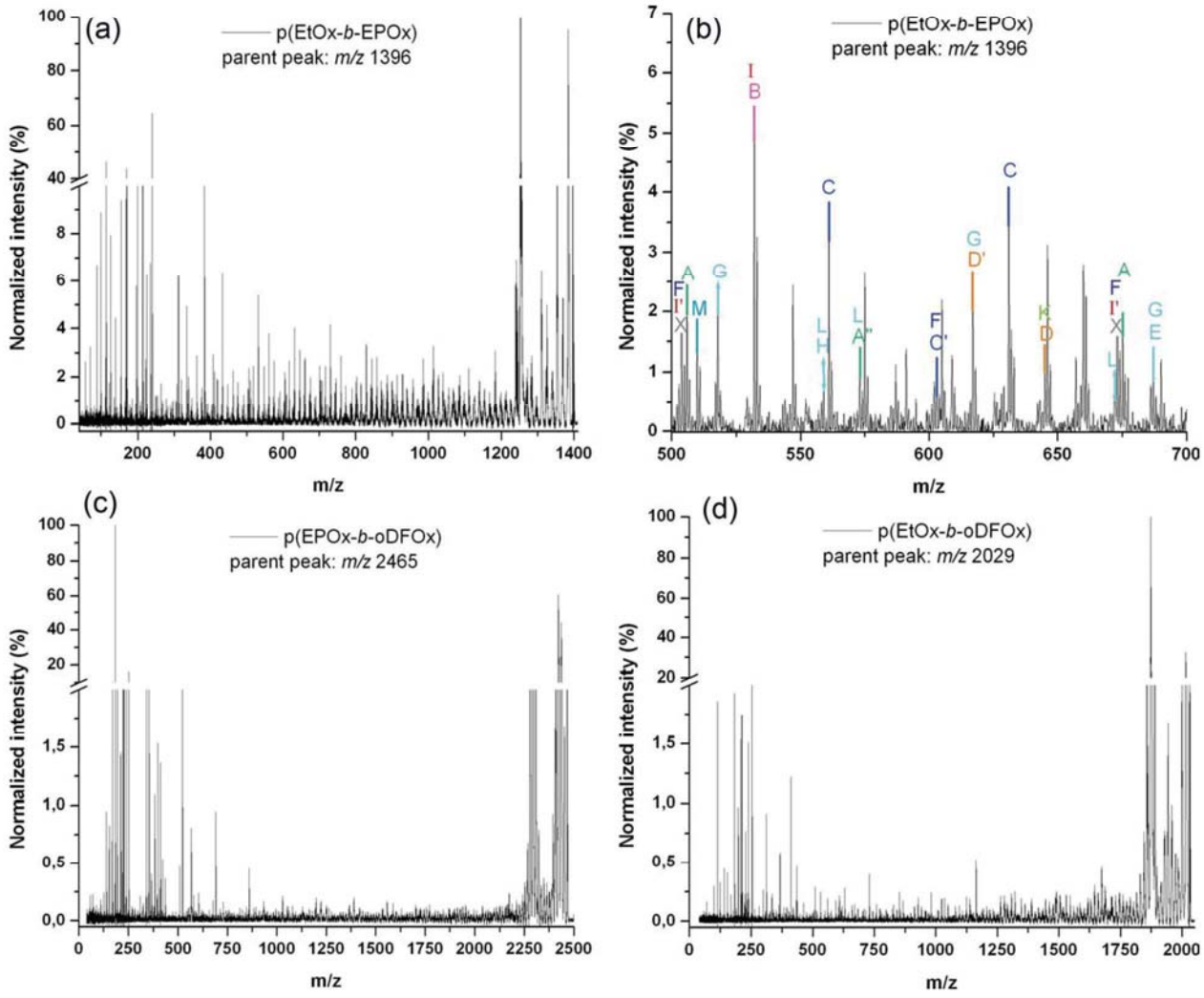


FIGURE 2 MALDI-TOF MS/MS spectra of different poly(2-oxazoline) block copolymers: (a) p(EtOx-*b*-EPOx) (parent peak: *m*/*z* 1396), (b) zoom into the MS spectrum of p(EtOx-*b*-EPOx) (*m*/*z* 500–700), (c) p(EPOx-*b*-oDFOx) (parent peak: *m*/*z* 2465), and (d) p(EtOx-*b*-oDFOx) (parent peak: *m*/*z* 2029).

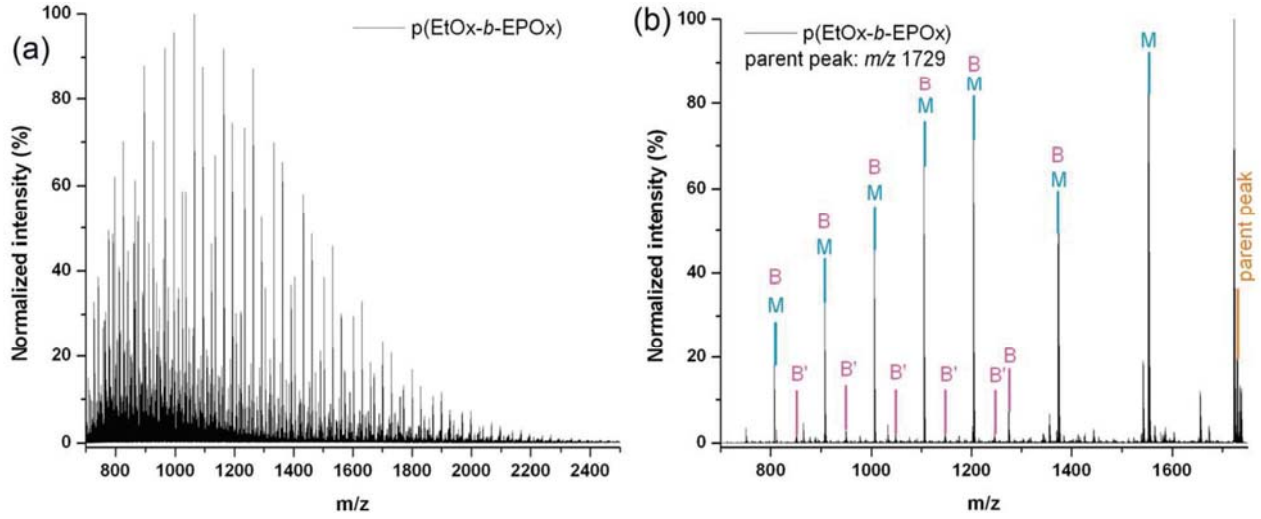
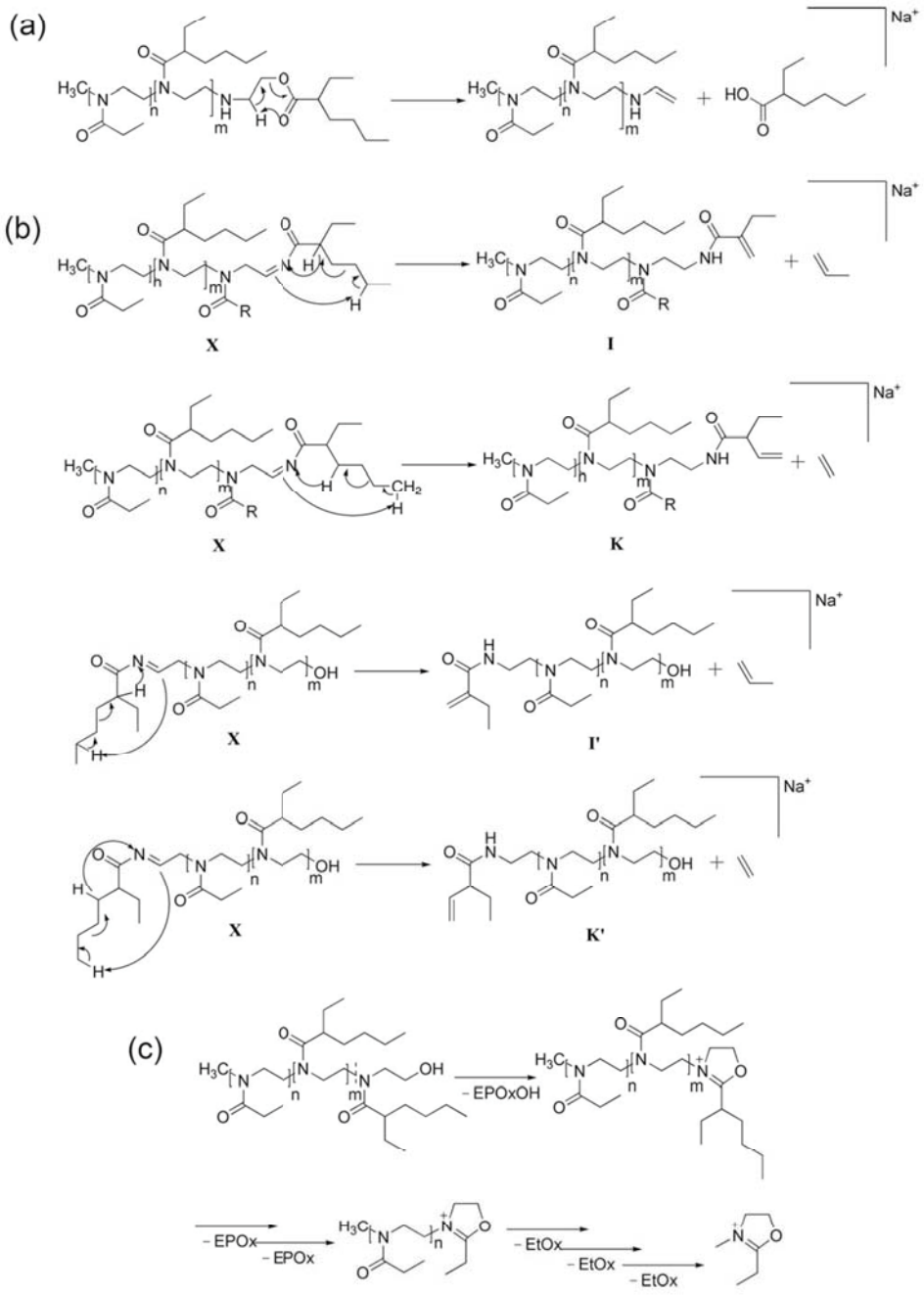


FIGURE 3 ESI-Q-TOF MS (a) and MS/MS (b) spectra of p(EtOx-*b*-EPOx).



SCHEME 2 Schematic representations of the possible fragmentation mechanisms occurring under MALDI-TOF MS/MS or ESI-Q-TOF MS/MS investigations: (a) McLafferty +1 rearrangement (MALDI); (b) alkyl elimination inside the 1-ethyl-pentyl side group (MALDI); and (c) depolymerization (ESI).

(EtOx) as first building block and five repeating units of 2-(1-ethyl-pentyl)-2-oxazoline (EPOx) as second building block. The initiating group was methyl and the terminating group can be either hydroxyl or $NHCH_2CH_2OCOC_7H_{15}$. In Figure 2(b) between m/z 500 and 700, most of the signals could be assigned to several possible fragmentation series. Due to the large number of fragmentation possibilities and the larger distance between the repeating units, only some of the later mentioned fragments are visible in Figure 2(b). The mass difference between the oligomers in these series can be either 99.07 m/z (2-ethyl-2-oxzaoline) or 169.15 m/z (EPOx) or both due to the fragmentation at different parts of the polymer chain. The 1,4-hydrogen or ethylene elimination via a six-membered transition state was one of the fragmentation mechanisms that could be found for p(EtOx) or PEG, which have both the same ethylene spacing group between the heteroatoms and have been already characterized by MALDI-TOF MS/MS.[25,26,33,34] This fragmentation behavior occurs even for the p(EtOx-*b*-EPOx) block copolymer, starting on both sides of the polymer chain, and the series are assigned by **A**, **A′** for the 1,4-ethylene elimination and **B**, **B′** for the 1,4-hydrogen elimination. The intensity of the counter fragments labeled with **X** was relatively weak; as a consequence, only some signals of them could be assigned. In

addition, these counter fragments can dissociate further to the fragment series **D** and **D′**, which were formed by a second ethylene elimination. The McLafferty rearrangement,[32] which includes the heteroatom of the side group in the six-membered transition state, was an alternative fragmentation possibility to form the fragment series **A**, **A′** and **B**, **B′**. The fragment series **C** and **C′** can be explained by an α-cleavage of the backbone between the ethylene spacing group followed by a cleavage of the side group to form a methylene imine end group. Furthermore, for each double series, a third fragmentation series (**A″**, **B″**, **C″**, and **D″**) can be found with $NHCH{=}CH_2$ as possible end group that can be explained by the side product structure and its fragmentation via McLafferty +1 rearrangement. The schematic representation of the fragmentation mechanisms (1,4-ethylene or hydrogen elimination, α-cleavage followed by side group elimination, and the additional elimination of ethylene inside the side group) was already shown in a previous publication.[26] The combination of the already found mechanisms led to the fragment series **E**, **F**, **G**, **H**, and **L**. The series **E** is explainable by ethylene elimination on one side and hydrogen elimination on the other side. Ethylene elimination performed on both sides of the polymer chain led to the fragment series **F** and the α-cleavage followed by a side group cleavage on both sides to **L**. The other series **G** and **H** could be explained by α-cleavage and side group elimination on the one side and ethylene elimination on the other side for **G** or hydrogen elimination for **H**. Due to the longer side chain of the second monomer EPOx, further fragment series **I**, **I′** and **K**, **K′** could be assigned and are displayed in Scheme 2(b). These fragments can also be formed from the instable counter fragments **X** with an additional propylene elimination for **I**, **I′** (eight-membered transition state) and ethylene elimination in the pentyl chain for **K**, **K′** (eight-membered transition state) of the 1-ethyl-pentyl side group. All these fragment series could be assigned for the other poly(2-oxazoline) block copolymers MS/MS spectra of this study. The block copolymers containing only monomers with alkyl side groups showed additional fragment series, like **D**, **D′** or **I**, **I′** and **K**, **K′**, which are already discussed before. This fragmentation behavior was not observed for monomers with aryl side groups, which seemed to be more stable under the CID conditions.

As second MS characterization method, ESI-Q-TOF MS was chosen to verify the composition of these poly(2-oxazoline) block copolymers. For the detailed interpretation and comparison with the MALDI results, p(EtOx-*b*-EPOx) was selected as a representative sample and the MS spectrum is shown in Figure 3(a). Compared with MALDI-TOF MS, ESI-Q-TOF MS requires only low concentrations of the analyte and a suitable solvent system instead of identifying the right matrix/salt combination. The spectrum showed only singly charged signals with one proton or one sodium as positive charge; therefore, deconvolution after the measurement was not necessary. In comparison to the MALDI-TOF MS spectrum, the signal intensity within the copolymer distribution shifted slightly to the lower molar mass region; however, in both spectra, the same signals could be found. For the tandem MS investigations, the signal at m/z 1729 was chosen as precursor ion [Fig. 3(b)], which had no overlapping isotopic pattern from another signal [marked with a circle in Fig. 3(a)]. This signal consisted of 12 repeating units EtOx and three repeating units EPOx. The ESI tandem MS spectrum showed less fragmentation in comparison to the MALDI-TOF MS/MS spectrum. As main fragmentation series, the depolymerization of the block copolymer could be assigned with **M**. The first loss within the polymer chain can be explained by the cleavage of one EPOx monomer with the hydroxyl end group to form the positively charged polymer chain with an oxazolinium species as occurring end group.[26] Afterward, the depolymerization continued further to eight EtOx repeating units. Furthermore, the 1,4-hydrogen elimination could be identified as fragmentation series **B**, **B′** in the ESI tandem MS spectrum. Additional fragments depending on mechanisms found for the MALDI-MS/MS spectrum, like 1,4-ethylene elimination, McLafferty rearrangement, or α-cleavage followed by side group elimination, were not observed in the ESI tandem MS spectrum. The lower degree of fragmentation in the ESI-Q-TOF MS/MS spectra compared with the MALDI-TOF MS/MS spectra indicate that ESI is the softer ionization process for this class of polymer.

EXPERIMENTAL

Materials

EtOx (99%, Acros) was dried over barium oxide and distilled under argon prior to use. 2-(2,6-Difluorophenyl)-2-oxazoline (oDFOx) and EPOx were synthesized as described previously.[10,35] Methyl tosylate (98%, Aldrich, MeTos) was distilled and stored under argon. Acetonitrile (extra dry, Acros) was stored under argon.

The matrix *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB, Sigma–Aldrich), sodium iodide (Acros Organics), as well as the solvents chloroform and acetone (HPLC grade, Roth) were used as purchased.

Synthesis of the Poly(2-oxazoline) Block Copolymers

For the synthesis of the diblock copolymers poly(2-ethyl-2-oxazoline)-*b*-poly(2-(1-ethyl-pentyl)-2-oxazoline) p(EtOx)-*b*-p(EPOx), poly(2-ethyl-2-oxazoline)-*b*-poly(2-(2,6-difluorophenyl)-2-oxazoline) p(EtOx)-*b*-p(oDFOx), and poly(2-(1-ethyl-pentyl)-2-oxazoline)-*b*-poly(2-(2,6-difluorophenyl)-2-oxazoline) p(EPOx)-*b*-p(oDFOx), stock solutions of the initiator (methyl tosylate), solvent (nitromethane), and monomer (EtOx and EPOx, respectively) were prepared and each stock solution was divided over two vials. These vials were exposed to microwave radiation for a precalculated time to obtain near-quantitative conversions ($\ln [M]_0/[M]_t = 4$). One of each polymerization was quenched by the addition of water. Subsequently, the second monomer was added via a syringe to the second vial, which was subjected to microwave irradiation, analogs to the first step. Finally, the polymerizations were terminated by the addition of water. ^{1}H NMR spectroscopy was used to confirm the absence of residual monomer as well as to investigate the polymer composition. After removing the nitromethane under reduced pressure, the block copolymers were dissolved in chloroform and precipitated in ice cold diethyl ether.

Instrumentation

SEC was measured on a Shimadzu system equipped with a SCL-10A system controller, a LC-10AD pump, and a RID-10A refractive index detector using a solvent mixture containing chloroform, triethylamine, and isopropanol (94:4:2) at a flow rate of 1 mL/min on a PSS-SDV-linear M 5 μm column at room temperature. The system was calibrated with PS (370–67,500 g/mol) and PMMA (2,000–88,000 g/mol) standards.

MALDI-TOF MS measurements were performed with an Ultraflex III TOF/TOF (Bruker Daltonics, Bremen, Germany) equipped with a Nd:YAG laser and a collision cell. All spectra were measured in the positive reflector mode. For the MS/MS measurements (LIFT^{TM} mode),[36] argon was used as collision gas at a pressure of 2×10^{-6} mbar. The instrument was calibrated prior to each measurement with an external PMMA standard (m/z 410 or 2,500) from PSS Polymer Standards Services GmbH (Mainz, Germany) in the required measurement range. MS and MS/MS data were processed using Flex Analysis 3.0, PolyTools 1.12 (beta version), Data Explorer 4.0, and an isotope pattern calculator.

ESI-Q-TOF-MS measurements were performed using a micrO-TOF-Q II (Bruker Daltonics) mass spectrometer equipped with an automatic syringe pump (KD Scientific) for sample injection. The ESI source was operated at 4.5 kV and a desolvation temperature of 180 °C. The samples were introduced by a continuous flow (3 μL/min) of a chloroform/acetonitrile mixture. All MS spectra were obtained in the positive mode, and argon was used as collision gas for the MS/MS measurements. The instrument was calibrated in the m/z range 50–3,000 using an internal calibration standard (Tunemix), which is supplied from Agilent. MS and MS/MS data were processed via Bruker Data Analysis software 4.0.

Sample Preparation

For the MALDI sample preparation, the poly(2-oxazoline) block copolymers (10 mg/mL) in chloroform, DCTB in chloroform (30 mg/mL), and the doping salt (NaI) dissolved in acetone at a concentration of 100 mg/mL were used. The dried-droplet spotting technique[37] (matrix, salt, and analyte previously mixed together) was applied. For each sample, 1 μL of the mixture was spotted on a target plate.

For the ESI sample preparation, the poly(2-oxazoline) block copolymers were dissolved in a chloroform/acetonitrile mixture (1:4) with a concentration of 1–10 μg/mL. The addition of a sodium salt prior to the analysis was not required.

CONCLUSIONS

Different poly(2-oxazoline) block copolymers were characterized in detail by MALDI-TOF and ESI-Q-TOF tandem MS. The investigation with a CID cell for the fragmentation of poly(2-oxazoline) block copolymers led to numerous fragment series. Besides 1,4-eliminations of ethylene or hydrogen, McLafferty rearrangement and other mechanisms, the second possible structure for the precursor ion could be verified by a McLafferty +1 rearrangement with the ester as leaving group for all investigated block copolymers. The influence of the different side chains on the fragmentation led to several additional series for the alkyl side groups, which are not observable for the more stable aryl side groups. The usage of ESI-Q-TOF MS/MS as second characterization method delivered a different fragmentation behavior under CID conditions. The comparison of these two methods showed that they are complementary to each other and that ESI seems to be a softer ionization technique than MALDI for poly(2-oxazoline)s.

In addition to the previous study of poly(2-oxazoline) homopolymers, the knowledge of this analysis improves the understanding of the polymerization mechanisms. Furthermore, it will alleviate further investigations of different complex block and random copolymers to achieve the construction of a MS/MS fragment ion library for a faster and automated analysis.

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Characterization of poly(2-oxazoline) homo- and copolymers by liquid chromatography at critical conditions

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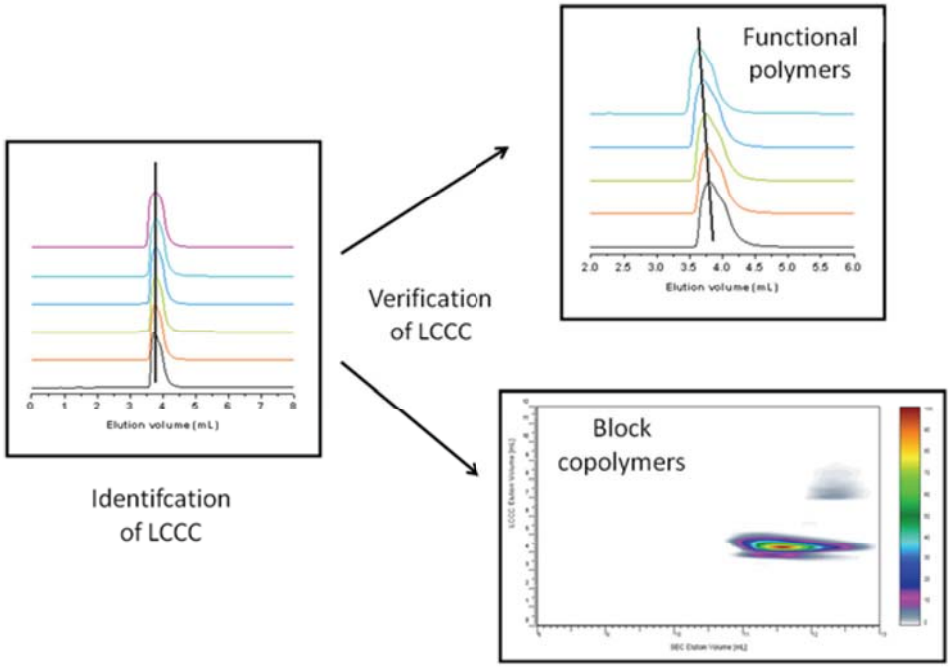

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Characterization of poly(2-oxazoline) homo- and copolymers by liquid chromatography at critical conditions

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ABSTRACT

In this study liquid chromatography at critical conditions for poly(2-ethyl-2-oxazoline)s (PEtOx) has been performed for the first time in order to analyze functional PEtOx homopolymers and block copolymers. Besides the verification of the critical point of adsorption with two series of ester end group functionalized PEtOx homopolymers, to evaluate the effect of both the chain length dependence and the end group polarity, using a cyano column with a solvent combination of 2-propanol and water, also two-dimensional liquid chromatography (2D-LC) has been applied for a poly(2-oxazoline) block copolymer. The combined characterization techniques provided further information about the polymerization procedure with regard to the formation of side-products by separation of the block copolymer from the corresponding homopolymer impurities. In addition, hyphenation of LCCC with MALDI-TOF MS and ESI-Q-TOF tandem mass spectrometry verified the obtained results.

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1. Introduction

Poly(2-oxazoline)s represent an interesting class of polymers due to the fact that they can be obtained by a living cationic ring-opening polymerization (CROP) enabling the synthesis of a wide range of well-defined (co)polymers [1–4]. During the last years, in particular water soluble poly(2-oxazoline)s, such as poly(2-ethyl-2-oxazoline) (PEtOx) and poly(2-methyl-2-oxazoline) (PMeOx), have received increasing attention since they are biocompatible and might be suitable candidates for the development of intelligent drug delivery systems [5]. Contemporary research on poly(2-oxazoline)s is focused on the synthesis and application of complex block copolymers [6–8]. However, conventional characterization methods, such as size exclusion chromatography (SEC), NMR spectroscopy or even mass spectrometry techniques, do not allow sufficiently detailed characterization if the methods are only applied as isolated tools. At this point the combination of different characterization techniques, such as two-dimensional chromatography at critical conditions or hyphenation of HPLC with MALDI-TOF MS or ESI-TOF MS, represents a highly valuable tool in order to deliver not only structural details, but also quantitative information about the copolymer and possible by-products. Such information is in particular required in cases where an application of poly(2-oxazoline) based copolymers in biological or medical fields is targeted.

Liquid chromatography at critical conditions (LCCC) represents a very powerful tool for the analysis of functional (co)polymers. With this straightforward technique it is possible to separate the polymer sample according to its chemical composition irrespective of its molar mass. The concept of the critical point of adsorption (CAP) was already described in the 1970s by Belenky et al. [9,10] and has been further developed by different groups [11–13]. By changing the solvent combination it is possible to alter the elution behavior of a homopolymer between the size exclusion mode (SEC mode), the liquid adsorption mode (LAC mode) and the transition mode or CAP on the same high-performance liquid chromatography (HPLC)

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column [14]. This attitude depends on the non-solvent/solvent combination at a constant measurement temperature and by variation of the solvent mixture the different modes can be reached. The theoretical background of this phenomenon was studied in detail by Gorbunov and Skvortsov [15,16] and can be described by the interaction parameter c and the Gibbs equation. In the SEC mode, the interaction of the sample and the stationary phase is driven by entropic forces resulting in a shorter retention time of polymers with higher molar mass. In contrast the enthalpic interactions are predominant in the LAC mode so that shorter polymer chains elute first [17]. At the CAP, the enthalpic and entropic interactions compensate each other, whereby the interaction parameter c equals zero. Therefore, all polymer chains become chromatographically "invisible" at the transition point and elute simultaneously independent of the molar mass enabling a separation of various copolymers according to their chemical composition only. In addition, the search for the transition point of a given polymer in HPLC is very time-consuming including the variation of several experimental parameters, such as the correct column material, the flow rate, the conditions for the evaporative light scattering detector (ELSD) as well as the column oven temperature and the "special" solvent combination [18]. If these parameters are not carefully fitted to the system, reduced or low sample recovery can occur as major drawback [19–21]. This very special characterization and separation technique was already applied to a range of block, star and graft-copolymers [17,22,23], functional polymers [24,25] as well as polymer blends [26]. However, to the best of our knowledge, no LCCC conditions of a poly(2-oxazoline) have ever been reported.

The coupling of LCCC with size-exclusion chromatography (SEC) [27] or different mass spectrometry techniques [28], such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) or electrospray ionization time-of-flight (ESI-TOF), represents a valuable method for the characterization of complex copolymer architectures. For this purpose, the aliquots from the LCCC dimension can be transferred to a SEC system in order to achieve simultaneous information about the structural heterogeneity as well as the molar mass distribution. This technical setup, designated as two-dimensional chromatography (2D-LC or LCCC × SEC), has been applied to different synthetic polymers in the last years [29–34]. Coupling of LCCC with a mass spectrometry technique is especially useful for polymers where no SEC standards are available and provides additional information about the polymer end groups. Using MALDI-TOF MS the coupling can be only applied offline by different spraying or spotting techniques [33,35]. In contrast, ESI-TOF MS can be coupled online to the 2D setup, which prevents problems that might occur during the MALDI-TOF sample preparation [28,36,37].

In this contribution, the critical conditions for poly(2-ethyl-2-oxazoline) (PEtOx) homopolymers were established for the first time and verified with a series of poly(2-ethyl-2-oxazoline)s with end groups of varying polarity. In addition, two-dimensional LCCC × SEC analysis and LCCC–MS was applied to a PEtOx based block copolymer.

2. Experimental

2.1. Materials

Unless specified otherwise, all solvents were obtained from standard suppliers and used without further purification. 2-Ethyl-2-oxazoline (EtOx) was purchased from Acros Organics and distilled over barium oxide under inert atmosphere prior to use. Acetonitrile (CH_3CN, extra dry) was purchased from Acros Organics and stored over molecular sieves under an argon atmosphere. Methyl tosylate (98%, Sigma Aldrich, MeTos) was distilled under reduced pressure and stored under argon. Triethylamine was dried over potassium hydroxide and distilled under argon. Acetic acid, butyric acid, hexanoic acid, decanoic acid and stearic acid were purchased from Sigma Aldrich and used as received. The components of the eluent for HPLC and SEC (2-propanol, water and tetrahydrofuran (THF), all HPLC grade), were obtained from Sigma Aldrich.

The MALDI-TOF MS matrix *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB, Sigma Aldrich), sodium iodide (Acros Organics) as well as the solvents chloroform and acetone (HPLC grade, Roth) were used as purchased.

2.2. General procedure for the synthesis of PEtOx standards and analytes

In a representative example for PEtOx-OAc with $M/I = 20$, MeTos (0.6 mmol, 112 mg), EtOx (12 mmol, 1.19 g) and CH_3CN (1.8 mL) were weighed into a pre-dried microwave vial under inert conditions. The concentration of monomer was kept at 4 mol L^{-1}. The vial was capped and heated to 140 °C using microwave irradiation [38]. After the desired reaction time (5 min for $M/I = 20$) the vial was cooled down automatically by a nitrogen flow. For the preparation of the standards with OH end groups 1 mL water was added to the vial. For the synthesis of PEtOx having ester end groups the desired acid was added via a syringe directly through the septum of the vial in 1.5-fold excess (for PEtOx-OAc: acetic acid 0.9 mmol, 52 μL). Decanoic and stearic acid were dissolved in 1 mL of dry dichloromethane and added in solution. Thereafter, triethylamine was added via syringe in 2-fold excess (1.2 mmol, 0.17 mL). The vials were placed in an oil bath at 70 °C overnight. After completion of the end capping procedure, samples for ^{1}H NMR spectroscopy were taken directly from the reaction mixtures in order to determine the conversion and the degree of polymerization (DP). For the purification of the polymers the reaction mixture was dissolved in chloroform and washed with saturated aqueous sodium hydrogen carbonate and with brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Finally, the polymers were precipitated into cold diethyl ether and dried under reduced pressure.

2.3. Synthesis of the poly(2-oxazoline) block copolymer

The synthesis of the diblock copolymer poly(2-ethyl-2-oxazoline)-*b*-poly(2-(2,6-difluorophenyl)-2-oxazoline) (p(EtOx)-*b*-p(*o*DFOx)) was reported elsewhere [39]. ^{1}H NMR spectroscopy was used to confirm the absence of residual monomer as well as to investigate the polymer composition.

2.4. Methods

The polymerization of 2-oxazolines was performed in a Biotage Initiator Sixty microwave synthesizer. ^{1}H NMR spectra were recorded on a Bruker Avance 300 MHz using the residual solvent resonance as an internal standard. For the measurement of the matrix-assisted laser desorption/ionization (MALDI) spectra an Ultraflex III TOF/TOF (Bruker Daltonics, Bremen, Germany) was used. The instrument was equipped with a Nd:YAG laser and a collision cell. All spectra were measured in the positive reflector or linear mode. The instrument was calibrated prior to each measurement with an external PMMA standard from PSS (Polymer Standards Service GmbH, Mainz, Germany). For the MALDI-TOF MS sample preparation, separate solutions of polymer (10 mg mL^{-1} in chloroform), DCTB (30 mg mL^{-1} in chloroform), and the doping salt NaI (100 mg mL^{-1} in acetone) were prepared and subsequently mixed according to the dried-droplet spotting technique [40]. For each sample 1 μL of the mixture was spotted onto a target plate. ESI-Q-TOF MS measurements were performed with a microTOF Q

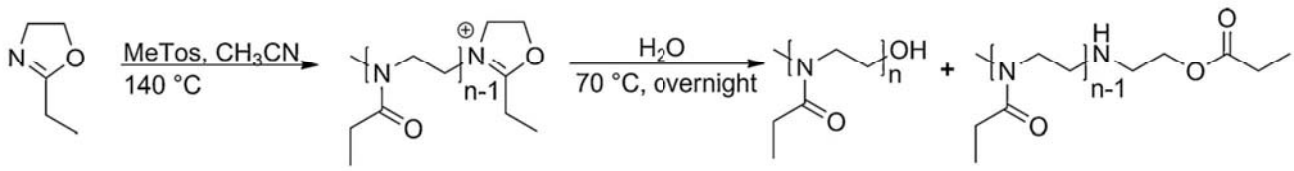


Fig. 1. Schematic representation of the synthesis of water quenched PEtOx-OH "standards".

(Bruker Daltonics) mass spectrometer equipped with an automatic syringe pump, which was supplied by KD Scientific. The mass spectrometer was operating in the positive ion mode. The standard electrospray ion (ESI) source was used to generate the ions. The ESI-Q-TOF-MS instrument was calibrated in the m/z range 50–3000 using an internal calibration standard (Tunemix solution), which was supplied from Agilent. Data were processed via Bruker Data Analysis software version 4.0.

2.4.1. Liquid chromatography under critical conditions (LCCC)

High-performance liquid chromatography (HPLC) was measured on an Agilent system (series 1200) equipped with a binary pump, an autosampler and an evaporative light scattering detector (ELSD, Softa Corporation, Model 400). For the LCCC separation, a Discovery Cyano column (Supelco, 250 mm × 4.6 mm, particle size 5 μm, pore size 180 Å) was used. The mobile phase consisted of a mixture of 2-propanol (IPA) and water (91/9, v/v) delivered by the binary pump at a flow rate of 1 mL min^{-1}. The column oven temperature was set to 35 °C. For the detection part, the ELSD was used with an evaporator temperature of 70 °C. The samples were dissolved at a concentration of 2 mg mL^{-1} in the same solvent mixture as the mobile phase, and for each measurement 50 μL were injected. Data were acquired using the WINGPC Unity software from PSS.

2.4.2. Size-exclusion chromatography (SEC)

To characterize the synthesized PEtOx homopolymers prior to 2D measurements, size exclusion chromatography (SEC) was measured separately on a Shimadzu system equipped with a SCL-10A system controller, a LC-10AD pump, and a RID-10A refractive index detector using a solvent mixture containing chloroform, triethylamine, and isopropanol (94:4:2) at a flow rate of 1 mL min^{-1} on a PSS-SDV-linear S column (PSS GmbH Mainz, 300 mm × 8 mm, particle size 5 μm) at 40 °C. The system was calibrated with polystyrene (M_n = 370–128000 g mol^{-1}) standards purchased from PSS.

2.4.3. Two-dimensional liquid chromatography (2D-LC)

For the first dimension LCCC of PEtOx, the analytical conditions were used as described above except that the flow rate was set to 0.1 or 0.05 mL min^{-1} in order to enable the subsequent SEC separation of the LCCC fractions for the 2D analysis. The different sample fractions of the LCCC separation were automatically transferred to the second dimension (SEC) via an eight-port valve system with 200 μL sample loops. On the SEC system, the fractions were separated on a SDV HighSpeed linear S column from PSS (50 mm × 20 mm, particle size 5 μm) using THF containing 0.07% triethylamine as eluent at a flow rate of 3 mL min^{-1} at 35 °C and the ELSD. For the 2D measurements higher concentrated polymer solutions (7 mg mL^{-1}) were prepared and 50 μL were used as injection volume. The data acquisition was done by the PSS WINGPC unity software, including a 2D software module.

3. Results and discussion

3.1. Identification of the critical conditions for PEtOx

Due to the lack of commercially available PEtOx polymer standards, which represent the basic material for identification of the critical conditions, the initial step of the present study comprised the synthesis of a series of suitable PEtOx homopolymers with varying molar masses. Fortunately, the cationic ring-opening polymerization (CROP) of 2-ethyl-2-oxazoline can be performed in a living manner enabling a straightforward access to well-defined polymers whose degree of polymerization (DP) and, therefore, molar mass is determined by the used ratio of [monomer] to [initiator] [38]. A series of PEtOx homopolymers with DP from 5 to 84 (corresponding to M_n values from 500 to 8400 g mol^{-1}) was synthesized using this strategy (Fig. 1), whereby the polymerization was terminated by addition of water to the reaction vial. All polymers were characterized by means of ^{1}H NMR spectroscopy, SEC, MALDI-TOF MS as well as ESI-Q-TOF MS and the obtained results are summarized in Table 1. As indicated by SEC measurements (see electronic supporting information (ESI) Fig. S.1) all polymers revealed narrow molar mass distributions with PDI values below 1.20. As commonly known, SEC, is a relative method, providing rough estimates of the M_n values as long as the correct standard polymer is not available. However, the DP of the PEtOx-OH standards could easily be calculated from the used M/I ratio and the monomer conversion when assuming the absence of chain transfer reactions. In addition, it could be obtained from the ^{1}H NMR spectra of the purified products using the peak integrals of the methyl group at the α chain end of the polymer (2.9 ppm) and the polymer backbone signal (3.3 ppm) again assuming that no transfer reactions occurred. Both values coincide well with one another as long as the molar mass remains rather low. However, with increasing molar mass an accurate integration of the peaks in the ^{1}H NMR spectra becomes more difficult resulting in a larger deviation of both values. Taking into account constraints of the applied method, such as ion selectivity or mass discrimination, also the M_n values obtained from MALDI-TOF MS analysis are in agreement with the theoretical

Table 1
Characterization data of the synthesized PEtOx-OH "standards".

	DP calc.	DP NMR	t_{pol}[a] [min]	$M_{n\ (SEC)}$ [g mol^{-1}]	PDI (SEC)	$M_{n\ (MALDI)}$ [g mol^{-1}]
P1	5.1	5.2	2	930	1.14	640
P2	9.4	9.5	3	1410	1.12	790
P3	18.5	17.9	5	2340	1.09	1390
P4	26.2	24.4	7	3030	1.11	2280
P5	45.8	40.2	12	4190	1.14	3310
P6	83.8	84.1	10[b]	6280	1.20	n/a

[a] t_{pol} denotes the reaction time of the polymerization.
[b] Conversion of P1–P5 is quantitative, conversion of P6 is 91%.

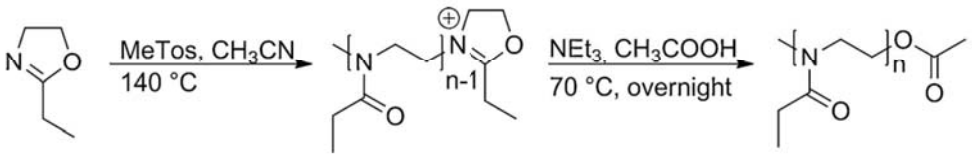


Fig. 2. Schematic representation of the synthesis of ester-end-functionalized PEtOx "standards".

Table 2
Characterization data of the synthesized ester end-functionalized PEtOx "standards".

	M/*I* calc.	DP NMR	t_{pol}[a] [min]	$M_{n\ (SEC)}$ [g mol^{-1}]	PDI (SEC)	$M_{n\ (MALDI)}$ [g mol^{-1}]
P7	4.8	5.1	2	790	1.16	650
P8	10.0	10.7	3	1400	1.13	1030
P9	18.9	16.9	5	2220	1.09	1650
P10	29.3	30.1	7	3050	1.12	2480
P11	39.8	46.0	10	3960	1.08	3490
P12	86.6	70.3	10[b]	5630	1.19	n/a

[a] t_{pol} denotes the reaction time of the polymerization.
[b] Conversion of P7–P11 is quantitative, conversion of P12 is 93%.

values for the lower molar mass standards. It should be noted that the termination of the polymerization by addition of water to the polymerization mixture may result in the formation of two types of end groups that are both depicted in Fig. 1 [41–43]. Indeed, initial HPLC measurements of those standards on a cyano column using methanol as eluent revealed two peaks in the chromatograms of all PEtOx-OH standards that are most likely caused by the presence of both end group types (see ESI Fig. S.2 for a representative chromatogram). This assumption is supported by MALDI-TOF MS and ESI-Q-TOF MS analysis of the fractionated eluate revealing identical distributions of both fractions as a result of the equal molar masses of both end groups (not shown). The presence of both end groups is also visible in the ^{1}H NMR spectrum (not shown).

Driven by the challenge to overcome the disadvantageous elution behavior of PEtOx-OH a series of new standards with more well-defined ω end groups was synthesized by direct end-capping of the living oxazolinium species with *in situ* deprotonated acetic acid (Fig. 2). The new PEtOx-OAc standards were characterized in a similar fashion as PEtOx-OH and the analytical data obtained for this second series are summarized in Table 2. SEC traces are provided in Fig. S.1 (ESI). As displayed in Fig. S.3 left (ESI), analysis by MALDI-TOF MS shows a single distribution that can be assigned to the desired polymer structure which is ionized by a sodium cation. In the ESI-Q-TOF MS a second distribution is observed due to the fact that the CH_3EtOx_nOAc structure is not only ionized by a sodium cation but also by a proton (see ESI Fig. S.3 right). In addition, analysis of the PEtOx-OAc standards by means of ^{1}H NMR spectroscopy (see ESI Fig. S.4, $n = 0$ for a representative example) revealed a quantitative functionalization of the ω end group, as indicated by equal integrals of the peaks derived from the methyl protons at both chain ends.

Subsequently, HPLC measurements of those new PEtOx-OAc standards were performed on columns with varying polarity, i.e. C8, C18 or CN columns, in order to screen the interaction of PEtOx-OAc with the column material using a range of suitable solvents for PEtOx-OAc homopolymers, such as THF, methanol or 2-propanol. Since the combination of a CN column from Supelco with 2-propanol as "good" solvent resulted in the best elution behavior of the polymer, further experiments were conducted by adding water as a non-solvent with a higher polarity index than 2-propanol in order to identify the critical point of adsorption. In order to determine the composition of the critical solvent mixture, HPLC measurements of P7–P12 were performed in eluents consisting of varying ratios of 2-propanol and water. The

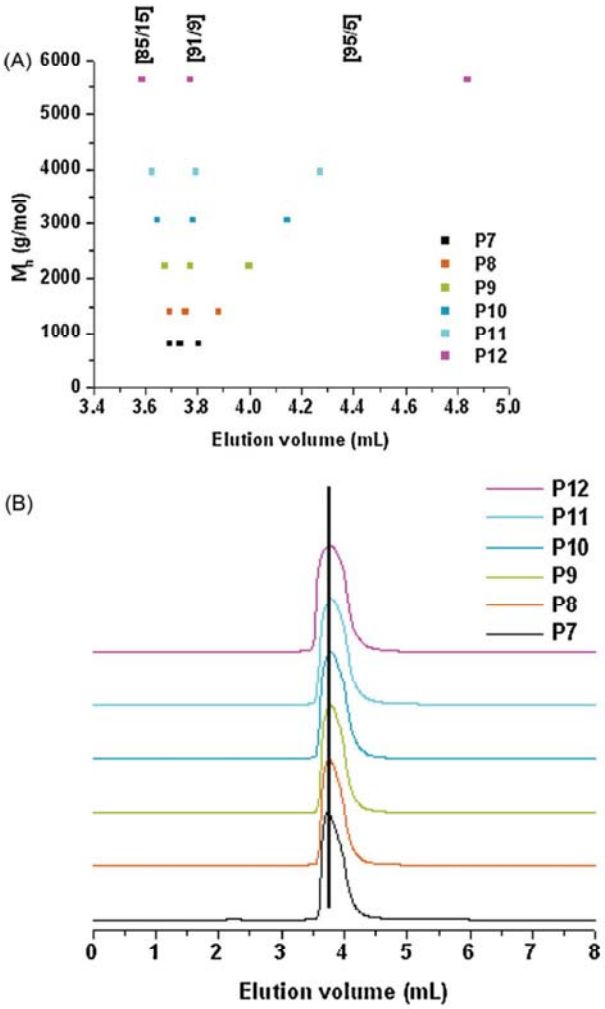


Fig. 3. (A) Elution volumes of a series of poly(2-ethyl-2-oxazoline) homopolymers P7 to P12 at various percentage of H_2O in 2-propanol as function of the polymer molar mass. (B) LCCC chromatograms of P7 to P12 "standards" at mobile phase composition 2-propanol/H_2O = 91/9 (v/v), i.e. critical conditions (ELSD evaporator temperature: 70 °C).

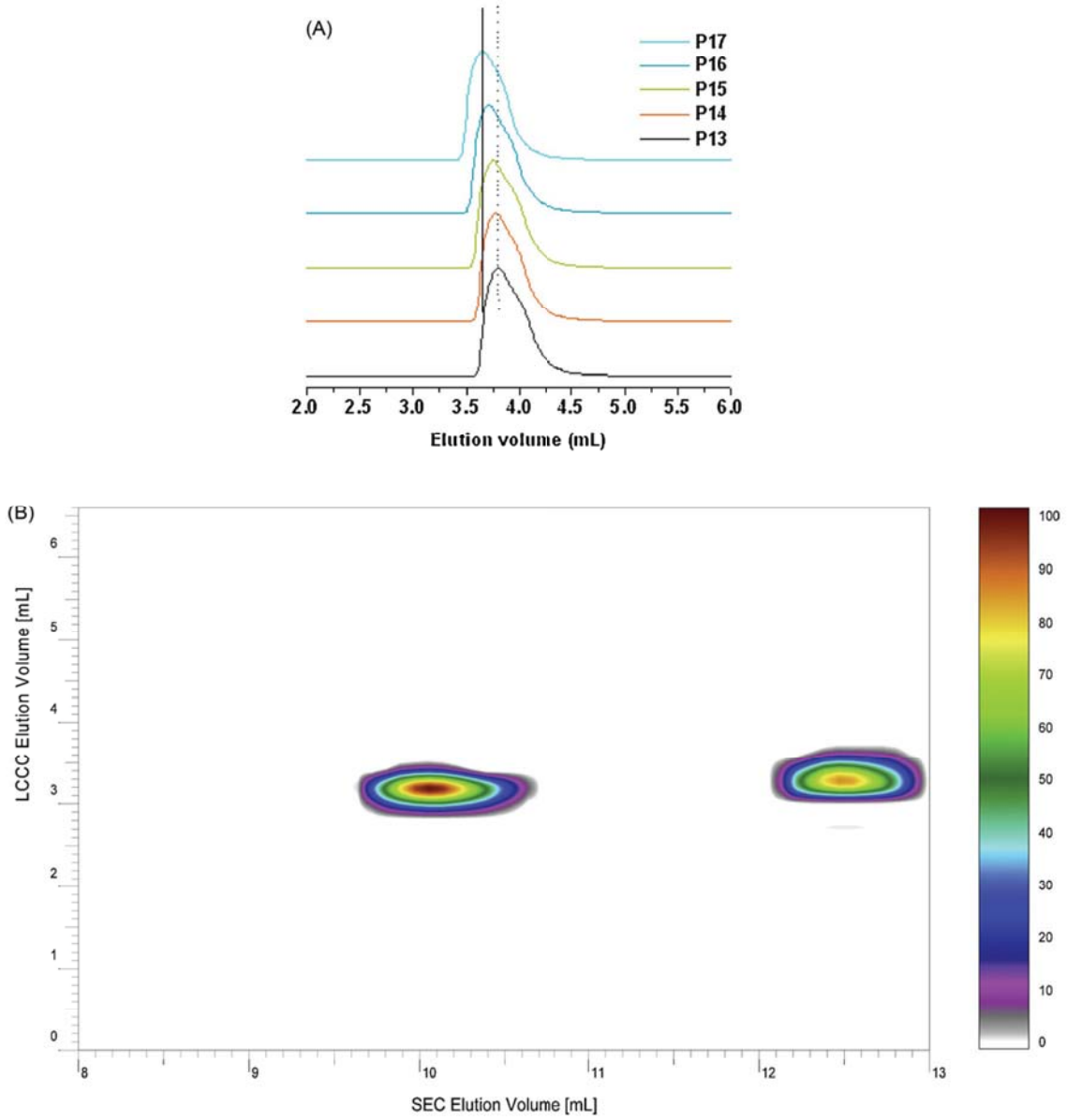
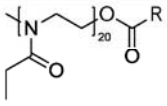


Fig. 4. (A) Elution chromatograms of P13 to P16 at the critical point of adsorption (CAP) (mobile phase composition: 2-propanol/H_2O = 91/9 (v/v); ELSD evaporator temperature: 70 °C). (B) 2D contour plot of mixed PEtOx homopolymers P7 and P12: 1st dimension LCCC for PEtOx (eluent: 2-propanol/H_2O = 91/9 (v/v)); 2nd dimension SEC (eluent: 0.07% triethylamine in THF).

Table 3
Characterization data of the synthesized PEtOx with varying end groups and schematic representation of their structure.



	R	$M_{n\ (SEC)}$ [g mol^{-1}]	PDI (SEC)	$M_{n\ (MALDI)}$ [g mol^{-1}]
P13	Methyl	2600	1.08	1550
P14	Propyl	2620	1.08	1800
P15	Pentyl	2600	1.08	1700
P16	Nonyl	2640	1.08	1960
P17	$C_{17}H_{35}$	2780	1.09	2010

Table 4
Characterization data of the synthesized stearic acid ester end-functionalized PEtOx with varying molar mass.

	M/*I* calc.	DP NMR	t_{pol}[a] [min]	$M_{n\ (SEC)}$ [g mol^{-1}]	PDI (SEC)	$M_{n\ (MALDI)}$ [g mol^{-1}]
P18	4.9	5.3	2	1290	1.12	950
P19	49.6	48	9.6	6560	1.09	5100

[a] t_{pol} denotes the reaction time of the polymerization.

corresponding elution volumes of the PEtOx-OAc standards with $M_{n,theo}$ from 500 to 8600 g mol^{-1} are depicted in Fig. 3(A). As can be clearly seen from the shift in the elution volume it was possible to change between the size-exclusion mode (where higher molar mass polymers elute first) using 85% 2-propanol and the adsorption mode (where shorter PEtOx-OAc elute first) with 95% 2-propanol. The critical conditions were reached at 91% 2-propanol whereby the elution volume for the critical point of adsorption could be identified at 3.79 mL (see Fig. 3(B)). In addition, the sample recovery decreased slowly by increasing molar mass of the PEtOx-OAc standards (see ESI Fig. S.5). These specific HPLC conditions should allow the characterization of a large range of synthetic (co)polymers comprising PEtOx by liquid chromatography at critical conditions (LCCC) or using 2D chromatography. Compared to other chromatographic methods that are applicable for the same task utilization of LCCC provides the advantage of a chromatographically "invisible" PEtOx part making the interpretation and peak assignment of the resulting chromatograms easier.

3.2. LCCC of PEtOx homopolymers with end groups of varying polarity

After observing the tremendous influence of the polymer end group upon its retention behavior on the HPLC column for PEtOx-OH homopolymers (see ESI Fig. S.2) it was interesting to study the end group effect in detail under critical conditions. In addition, such a study should represent a valuable tool for the verification of the critical conditions prior to performing 2D LCCC/SEC runs of copolymer analytes of uncertain composition. For this purpose, several PEtOx homopolymers with defined end groups covering a wide range of hydrophobicity was tailor made by end capping the living polymer chains with a homologous series of *n*-alkyl carboxylic acids from 1 to 18 carbon atoms, i.e. acetic acid, butyric acid, hexanoic acid, decanoic acid and stearic acid, respectively (compare Fig. 2). Since the influence of the end group upon LCCC is targeted the molar mass of the ester end functionalized PEtOx was kept rather low at a DP of 20.

The structures of these homopolymers (P13–P17) were characterized in an analogous fashion as the PEtOx standards and the results are summarized in Table 3. Analysis of P13–P17 by SEC (see ESI Fig. S.1) revealed narrow molar mass distributions with PDI values below 1.1 for all polymers, which could be confirmed by MALDI-TOF MS measurements. Increasing length of the alkyl moiety at the ω chain ends resulted in a slight shift to lower elution volume for P16 and P17. The successful functionalization with the desired end groups was confirmed by ^{1}H NMR spectroscopy (see ESI Fig. S.4) as well as by MALDI-TOF MS and ESI-Q-TOF MS (not shown). Integration of the peaks in the ^{1}H NMR spectra derived from the methyl protons at the α and ω chain ends of the polymers indicated a quantitative functionalization in all cases. Subsequently, HPLC measurements under critical conditions were performed in order to evaluate the effect of the polarity of the polymer end group on its retention behavior. As depicted in Fig. 4(A), the polymer containing a heptadecyl ester end group P17 elutes first (at 3.64 mL) and the polymer with a methyl ester end group P13 elutes last (at 3.79 mL). This observation is in agreement with the polarity of the used CN column: polymer end groups of lower polarity (longer alkyl chains) should interact less than those consisting of shorter alkyl chains. In order to verify if the observed retention behavior was simply caused by a SEC effect of the longer alkyl chains, two additional homopolymers (P18 and P19) with a heptadecyl end group were synthesized, one of them having a lower and the other one having a higher molar mass than P17 (Table 4). As depicted in Fig. S.6 (ESI), all three homopolymers with the same heptadecyl end group elute at the same retention volume from the column irrespective of their molar mass. These results clearly demonstrate that the

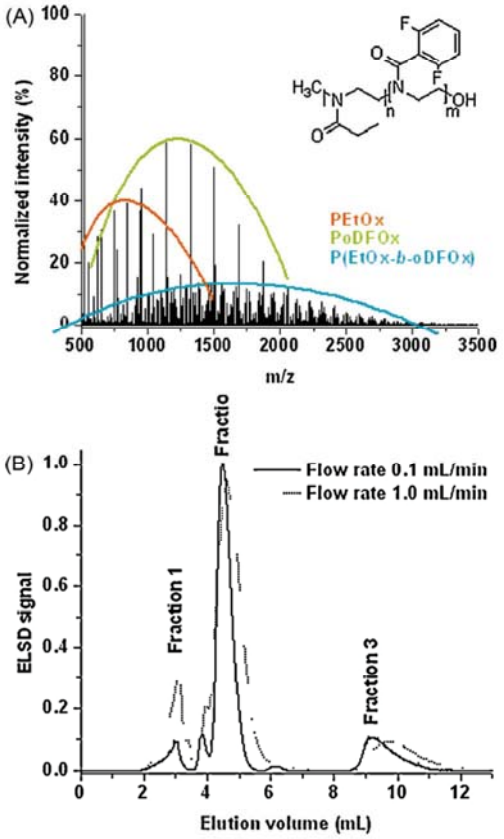


Fig. 5. (A) MALDI-TOF MS spectrum of the P(EtOx-*b*-*o*DFOx) copolymer. (B) Chromatograms of the P(EtOx-*b*-*o*DFOx) copolymer at CAP comparing different flow rates (indication of the fractionation process by lines).

retention time of the PEtOx homopolymers at the CAP of LCCC depend only on the end-group functionality (see ESI Fig. S.6).

In order to validate the separation of PEtOx-OAc standards with different M_n values in the SEC dimension of a 2D-LC setup prior to analysis of a block copolymer sample, liquid chromatography at critical conditions coupled with size-exclusion chromatography (LCCC × SEC or 2D-LC) was performed for a mixture of P7 and P12. As depicted in Fig. 4(B), the standards could be separated in the SEC dimension after their elution at the CAP in the LCCC dimension. Based on these experiments, the critical point of adsorption for PEtOx using this special column and the solvent composition of 9% water in 2-propanol could be proven in a straightforward fashion.

3.3. LCCC × SEC (2D-LC) analysis of a poly(2-ethyl-2-oxazoline) containing block copolymer

During the synthesis of poly(2-ethyl-2-oxazoline)-*b*-poly(2-(2,6-difluorophenyl)-2-oxazoline) P(EtOx)-*b*-p(*o*DFOx) block copolymers using the CROP technique the formation of homopolymers from both monomers as side products is possible, which results in a complex polymer mixture. The underlying termination and chain transfer reactions that might occur during the CROP are illustrated in Fig. S.7 (ESI) [44]. PEtOx homopolymer may be formed either by termination or chain transfer reactions during the polymerization of the first monomer, whereas the PoDFOx homopolymer can only originate from chain transfer processes during the polymerization of the second monomer [45]. The existence of both homopolymers could already be confirmed by careful analysis of the MALDI-TOF MS spectrum (Fig. 5(A)). As a result of termination reactions due to the presence of water

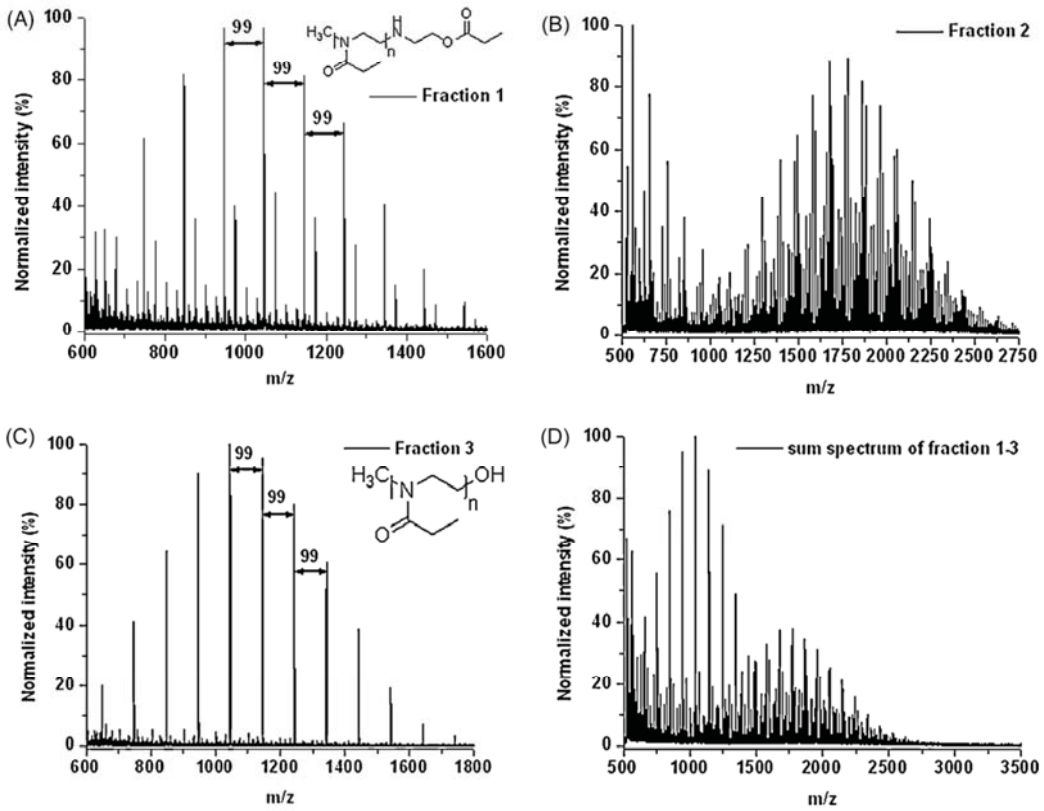


Fig. 6. MALDI-TOF MS spectra of the fractionated copolymer samples from HPLC measurement: (A) fraction 1 (elution volume: 2.2–3.6 mL); (B) fraction 2 (elution volume: 3.8–6.2 mL); (C) fraction 3 (elution volume: 8.0–10.5 mL); (D) accumulated spectrum of fraction 1–3.

during the polymerization, the PEtOx homopolymer (marked in red) can be assigned to the end groups $CH_3(C_5H_9NO)_{n-1}OH$ or $CH_3(C_5H_9NO)_nNHCH_2CH_2OCOCH_2CH_3$. On the other hand, since PoDFOx is formed by chain transfer reaction, the corresponding distribution can be assigned to proton initiated chains ($H(C_9H_7NOF_2)_mOH$; marked in green). The desired block copolymer is marked blue in Fig. 5(A). Unfortunately, the amount of these homopolymers cannot be quantified by MALDI-TOF MS as characterization technique, whereas liquid chromatography under critical conditions coupled with size exclusion chromatography (LCCC × SEC) is a perfect tool for this task, if the ELSD signal depends linearly on the concentration. However, since the response factors of the ELSD were not determined, the following interpretation of the 2D contour plot is only semi quantitative. In this approach, the separation takes place with regard to the chemical composition of the polymer by using 2-propanol/H_2O (91/9, v/v) as eluent mixture in the first dimension as well as with regard to the molar mass in the second dimension. Prior to the analysis of P(EtOx)-*b*-p(oDFOx) by two dimensional chromatography, the block copolymer was analyzed by one dimensional LCCC utilizing flow rates of 1.0 mL min^{-1} (as applied for the analysis of homopolymers) and 0.1 mL min^{-1} (the flow rate that is applied during a 2D LCCC × SEC run), respectively. For a better comparison of the spots occurring in the 2D contour plot, the same flow rate of 0.1 mL min^{-1} was chosen for the single LCCC analysis. The resulting chromatograms in Fig. 5(B) showed more or less similar elution behavior; although the lower flow rate (solid line) revealed an additional signal at 6 mL, which is probably disguised in the more pronounced tailing at higher flow rates (dotted line), assuming more time for column-analyte interactions at lower flow rates. With regard to the peak assignment of the chromatogram it should be noted that the PoDFOx homopolymer is insoluble in the applied eluent mixture due to its hydrophobic character preventing its elution over the column, which also obstructs quantification of the total amount of side products present in the block copolymer. In addition, the polymerization of the P(EtOx)-*b*-p(oDFOx) block copolymer was quenched by addition of water resulting in similar mixed ω end groups as for the PEtOx-OH standards P1–P6. Based on the experience gained during the HPLC analysis of the standards P1–P6, whose elugrams revealed two peaks (see ESI Fig. S.2 right), a possible peak assignment might be the following: The first signal (at 3 mL) belongs to a PEtOx homopolymer with an ester end group because its retention time is close to the critical point of adsorption for PEtOx with a methyl ester end group. The copolymer elutes around 5 mL represented by the main bimodal peak. The last signal around a retention time of 8.5 mL can probably be assigned to the second PEtOx homopolymer species with hydroxy (OH) as end group assuming a longer interaction time of this end group with the polar column material.

In order to confirm these assumptions the mobile phase of the LCCC run was fractionated. Therefore, three fractions were taken (2.2–3.6 mL, 3.8–6.2 mL and 8.0–10.5 mL) and the experiment was repeated several times to collect a sufficient amount of material for analysis by MALDI-TOF and ESI-Q-TOF MS. As shown in Fig. 6(A)–(C) for MALDI-TOF MS, the fractionation yielded nicely separated homo- and copolymers. In fraction 1 as well as fraction 3, only two kinds of PEtOx homopolymers could be identified, whereby fraction 2 consists only of the P(EtOx)-*b*-p(oDFOx) block copolymer. The main *m*/*z* distributions of fractions 1 and 3 can be assigned to the following structures $CH_3(C_5H_9NO)_nOH$ or $CH_3(C_5H_9NO)_{n-1}NHCH_2CH_2OCOCH_2CH_3$, which result in the same *m*/*z* value but correspond to different structures due to their different interaction efficiency with the used column material. Unfortunately, no meaningful structures could be assigned to the other distributions occurring in fraction 1. The accumulation of the three different MS spectra delivered an overview spectrum

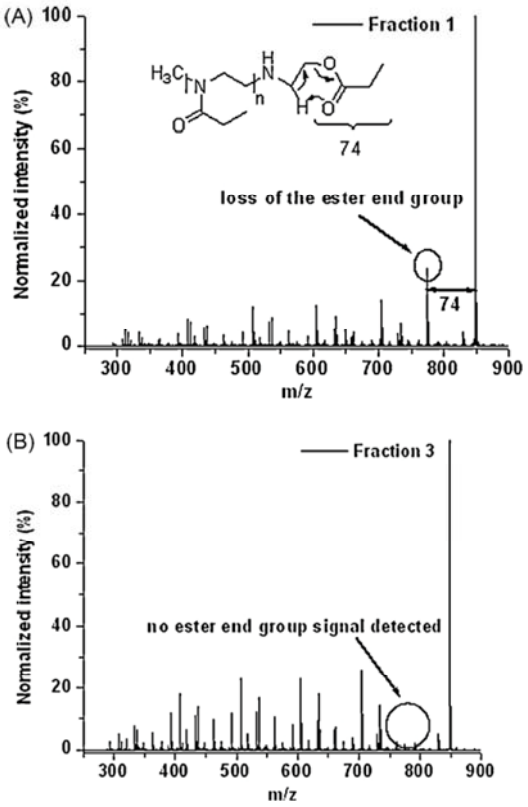


Fig. 7. ESI-Q-TOF MS/MS spectra of the fractionated copolymers samples from HPLC measurements: (A) fraction 1; (B) fraction 3.

(see Fig. 6(D)) similar to the MS spectrum of the unfractionated copolymer sample (see Fig. 5(A)) without the second homopolymer distribution of PoDFOx due to its insolubility in 2-propanol/H_2O. Unfortunately, the clear identification of the main distributions of fractions 1 and 3 by a single MALDI-TOF MS analysis interpretation is not possible because both end groups that may be present have the same molar mass. However, it should be feasible to distinguish between a hydroxyl and an ester-based end group by tandem MS measurements due to their different fragmentation behavior during collision induced dissociation. Due to the softer ionization the MS/MS analysis was done by ESI-Q-TOF MS. The results are displayed in Fig. 7(A) and (B). Comparing these tandem MS spectra, only in Fig. 7(A) the cleavage of the ester end group by an 1,5-hydrogen rearrangement is visible (marked with a circle). That indicates a parent peak structure with an ester end group ($CH_3(C_5H_9NO)_{n-1}NHCH_2CH_2OCOCH_2CH_3$) for fraction 1. The fact that this fragmentation is not visible in the tandem MS of fraction 3 supports the assumption of the presence of a hydroxyl end group, which could also be confirmed by the interpretation of the different fragmentation series displayed in Fig. 7(B) (similar to series in Refs. [46,47]). Based on these additional MS measurements, the interpretation of the previous LCCC analysis could be verified.

Finally, the LCCC × SEC analysis of the P(EtOx)-*b*-p(*o*DFOx) block copolymer was performed in order to gain additional information on the molar mass of the components inside the copolymer sample. By the use of an eight-port transfer valve, single 200 μL fractions of the LCCC run were transferred to a SEC high speed column. This analysis technique yielded a distribution with information on the chemical heterogeneity on the ordinate and the molar mass distribution on the abscissa. The 2D results of the copolymer analysis are shown in the contour plot in Fig. 8. By comparison with the previous LCCC measurement the main signal at 4.5 mL belongs to the copolymer and the other two signals represent the PEtOx homopolymers with different end groups. Furthermore, the "egg-like" shape of the copolymer spot can be explained by the existence of smaller copolymer chains, which can be clearly found in the MALDI-TOF MS spectrum of fraction 2 (see Fig. 6(B)) as well as in the LCCC chromatogram using a flow rate of 0.1 mL min^{-1} (see Fig. 5(B)). When comparing the V_{el} of the fractions in the SEC dimension it is obvious that both homopolymer peaks would overlap with the copolymer peak in an one dimensional SEC measurement stressing the necessity of 2D measurements for a complete characterization

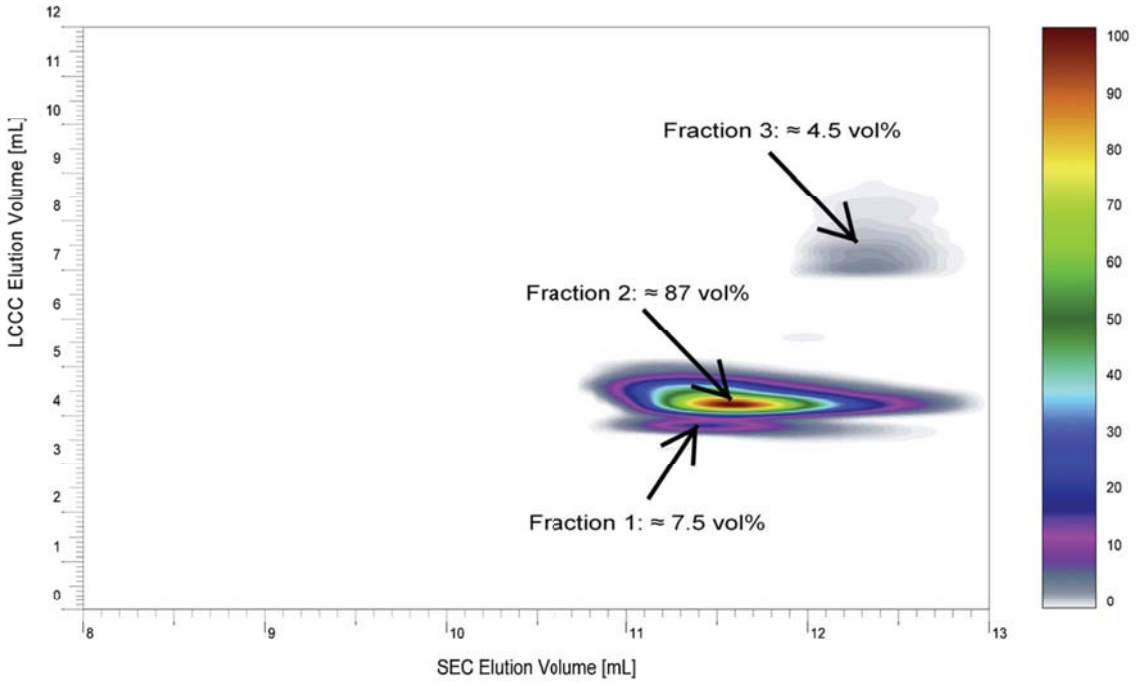


Fig. 8. 2D contour plot of the P(EtOx-*b*-*o*DFOx) copolymer: 1st dimension LCCC for PEtOx (eluent: 2-propanol/H_2O = 91/9 (v/v)); 2nd dimension SEC (eluent: 0.07% triethylamine in THF).

of such block copolymers. In addition, fraction 3 seems to correspond to a PEtOx homopolymer of lower molar mass than fraction 1. One might speculate that the effect of the terminal hydroxyl group on the elution behavior of the entire polymer in the HPLC dimension is more pronounced for shorter polymer chains. Nevertheless, it is clearly visible from the contour plot that the copolymer amount is predominant in comparison to the homopolymer spots. The manual interpretation by WINGPC indicated the presence of around 87.6% of block copolymer and around 7.5% and 4.5% of PEtOx homopolymer fractions 1 and 3, respectively. These semiquantitative results show clearly that the existing homopolymers are only side products of the polymerization, which could not be seen from the MALDI-TOF MS spectrum alone due to the depression of the copolymer in comparison to the homopolymers during the measurement. Nonetheless, the presence of 12% PEtOx homopolymer impurity together with an unknown amount of PoDFOx homopolymers impurity represents a significant amount indicating that the block copolymer is not as well-defined as previously concluded based on the 1D analysis.

4. Conclusions

In this study, the critical conditions for a poly(2-oxazoline), namely poly(2-ethyl-2-oxazoline), were established for the first time. The critical point of adsorption could be identified for this polymer class using a mixture of 2-propanol/H_2O as eluent on a cyano column. By varying the solvent composition it was possible to change between the adsorption mode, the exclusion mode and the critical point of adsorption. LCCC analysis was successfully applied for a tailored series of PEtOx homopolymers with end groups of varying polarity as well as for a block copolymer comprising PEtOx and a fluorinated block. Hyphenation of LCCC with MALDI-TOF MS and ESI-Q-TOF tandem mass spectrometry enabled the full assignment of all PEtOx chain transfer and termination products that were present in the block copolymer sample and their amount could be quantified by application of 2D chromatography with SEC in the second dimension.

These results represent a versatile basis for the detailed analysis of a wide range of functional copolymers containing PEtOx by liquid chromatography under critical conditions and two-dimensional chromatography, which is certainly required with respect to future application of this polymer class in bio-medical fields.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.09.044.

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Recent developments in the detailed characterization of polymers by multidimensional chromatography

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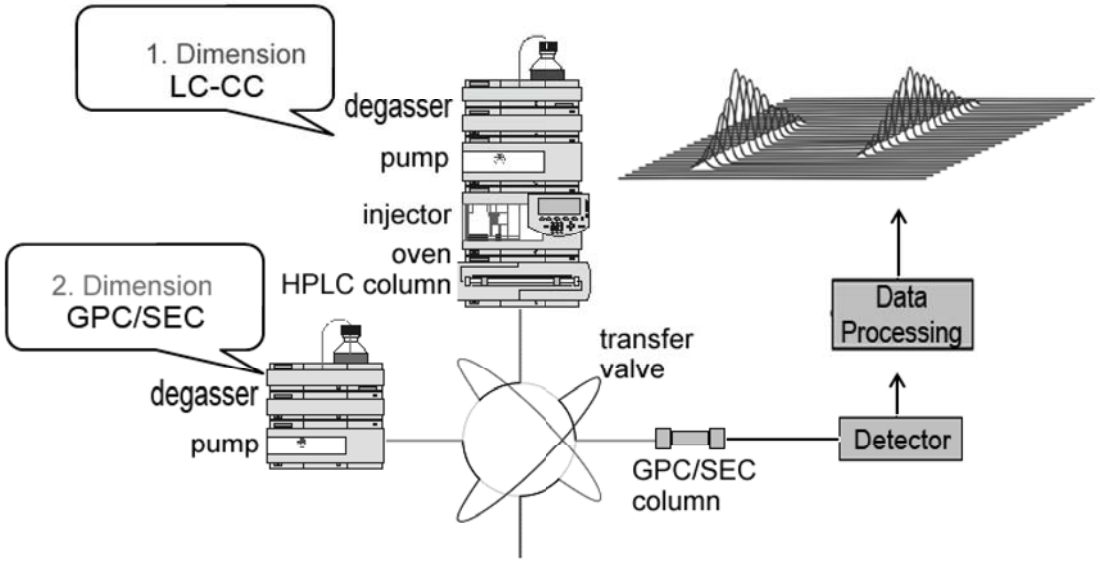

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Review

Recent developments in the detailed characterization of polymers by multidimensional chromatography

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ABSTRACT

Synthetic polymers as well as biopolymers reveal complex structures, such as variations in functionality, chain length and architecture. Therefore, combinations of different chromatographic techniques are a prerequisite for a detailed characterization. One possible approach is the combination of high performance liquid chromatography at critical conditions (LCCC) and size-exclusion chromatography, also named as two-dimensional chromatography, which allows the separation of the polymers according to different properties, like molar mass, chemical composition or functionality. In addition, LCCC hyphenated with different mass spectrometry techniques, *e.g.* MALDI-TOF or ESI-TOF, leads to additional information about molecular details of the polymeric structure. We summarize in this article the recent developments in two-dimensional chromatography of synthetic polymers and biopolymers since 2005.

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Contents

Abbreviations: ^{1}H NMR, nuclear magnetic resonance spectroscopy; 2D-LC, two-dimensional liquid chromatography; AGET, activators generated by electron transfer; APCI, atmospheric pressure chemical ionization; APE, alkyl phenol ethoxylate; ATRP, atom transfer radical polymerization; CAP, critical point of adsorption; CCD, chemical-composition dispersity; CID, collision-induced dissociation; DMF, dimethylformamide; ELSD, evaporative light scattering detector; EP, ethylene-propylene; EPDM, ethylene-propylene-diene; ESI-TOF MS, electrospray ionization mass spectrometry; ESR, electron spin resonance; EVAc, ethylene-vinyl acetate; FBMA, 2,2,3,3,4,4,4-heptafluorobutyl methacrylate; FTD, functionality-type dispersity; FT-IR, Fourier transform infrared spectroscopy; GPEC, gradient polymer elution chromatography; HILIC-PLOT, hydrophilic porous layer open tubular; HPLC, high-performance liquid chromatography; HPMC, hydroxylpropylmethylcellulose; HT 2D-LC, high-temperature two-dimensional chromatography; HT-SEC, high-temperature size-exclusion chromatography; LAC, liquid adsorption chromatography; LEAC, liquid exclusion adsorption chromatography; LC, liquid chromatography; LCCC, liquid chromatography at critical conditions; LC LCD, liquid chromatography under limiting conditions of desorption; LSSM, linear solvent strength model; MA, methacrylate; MALDI-TOF MS, matrix-assisted laser desorption/ionization mass spectrometry; MEK, methyl ethyl ketone; MMA, methyl methacrylate; MMD, molar-mass dispersity; MS/MS, tandem mass spectrometry; MTF, molecular-topology fractionation; NMP, nitroxide-mediated polymerization; NPLC, normal phase liquid chromatography; PAMAM, poly(amidoamine); PB, poly(butadiene); PBA, poly(butyl acrylate); PBMA, poly(butyl methacrylate); PBO, poly(butylene oxide); PBT, poly(butylenes terephthalate); PC, poly(carbonate); PCL, poly(ε-caprolactone); PDI, polydispersity index; PDMS, poly(dimethylsiloxane); PE, poly(ethylene); PEO, poly(ethylene oxide); PET, poly(ethylene terephthalate); PEtOx, poly(2-ethyl-2-oxazoline); PHO, poly(hexylene oxide); PI, poly(isoprene); P*i*BoA, poly(*iso*bornyl acrylate); PM, polymer model; PMAA, poly(methacrylic acid); PMMA, poly(methyl methacrylate); POc, poly(octene); PP, poly(propylene); PPO, poly(propylene oxide); PS, poly(styrene); PVAc, poly(vinyl acetate); PVAlc, poly(vinyl alcohol); Py-GC MS, pyrolysis-gas chromatography mass spectrometry; QSSM, quadratic solvent strength model; RAFT, reversible addition fragmentation chain transfer; RPLC, reversed phase liquid chromatography; RP-TGIC, reversed phase temperature gradient interaction chromatography; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TREF, temperature rising elution fractionation; UPLC, ultra high performance liquid chromatography; VP, N-vinylpyrrolidone.

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1. Two-dimensional chromatography

Combining two independent chromatographic techniques to separate polymer samples selectively with respect to either molar mass, chemical composition or functionality is termed as two-dimensional chromatography. First investigations in the field of two-dimensional chromatography including the principle of the sample dimensionality, which is based on the difference between ordered and disordered dispersities of component peaks in separation systems in relation to the sample complexity, were performed by Giddings in the early 1990s [1,2]. He described in detail the problematic and theoretical aspects regarding the selection of the most suitable multidimensional separation system. The theory behind two- or multidimensional chromatography for polymers and different coupled chromatographic methods are the main topic of several reviews in the last six years, whereby the application for the characterization of polymers is described only as subtopic. The column selectivity for two-dimensional liquid chromatography (2D-LC) was discussed by Jandera with special attention to multidimensional samples with various types of repeating units [3]. General aspects for the development of comprehensive multidimensional liquid chromatography as well as on the applications in different areas, such as natural, biological, pharmaceutical or organic compounds, were summarized by Dugo et al. [4], Guiochon et al. [5], Kivilompolo et al. [6], van der Horst et al. [7] as well as by Rittig and Pasch in "Multidimensional Liquid Chromatography in Industrial Applications" [8]. An overview about the theoretical aspects and the practical considerations for a LC × LC method development was described by François et al. in detail [9]. In 2010, Berek focused in his review on the principles of 2D-LC and the technical coupling concepts for low-molecular and polymeric substances [10]. Furthermore, the optimization of the 2D-LC method for non-proteomic applications is the main topic of the progress report written by Stoll [11]. In all these review articles, at least a minimum of two different and independent characterization methods has to be used to reach a complete analysis of a complex polymer sample (for a theoretical example see Fig. 1). In contrast to all the published summaries covering the theoretical background of combined characterization methods, this review provides an overview about the application of liquid chromatography at critical conditions (LCCC) as well as for other liquid chromatography methods for synthetic polymers and biopolymers. The integration of combined characterization techniques, *e.g.* LCCC hyphenated with size-exclusion chromatography, liquid chromatography or mass spectrometry, as analysis methods in this article will elucidate the advantages of coupled techniques.

The combination of high-performance liquid chromatography (HPLC) and size-exclusion chromatography (SEC) allows in most cases to achieve a sensitive separation according to chemical composition or functionality type on one hand and to molar mass on the other hand. These combined chromatographic techniques can be either performed in an off-line or an on-line approach [12]. Off-line separation, also called "cross-fractionation or heart-cut" method, can be performed by collecting the major fractions of the first dimension and reinjection to the second dimension. This very time, labor-intensive and energy consuming technique was replaced in the 1970s by Erni and Frei by a comprehensive two-dimensional LC method [13]. In this on-line approach, or better known as comprehensive approach, equal aliquots of the first dimension column effluent are transferred *via* an automated switching valve to the second dimension [7]. A typical comprehensive 2D setup is shown in Fig. 2. The theoretical aspects of 2D-LC including the different chromatographic modes of column separation as well as an overview about the diverse detection techniques for the SEC as second dimension is provided by Held and Kilz in their publication about the characterization of polymers by liquid chromatography [14]. New developments in the 2D polymer analysis to enhance the analysis time were investigated by Adler and Kilz [15]. Further setup constructions were done by Vanhoutte et al. to establish a spatial comprehensive 2D-LC system for high-pressure applications [16].

As can be seen from Fig. 2, one major chromatographic technique for the first dimension is LCCC. Applying this powerful separation method, functionalized homopolymers as well as different copolymers, such as blocks or star-shaped ones, can be separated from each other or from possible by-products. In this separation procedure, the separation according to molar mass is suppressed. The theoretical background of the critical conditions for one of the copolymer components as well as the interaction conditions close to the critical point or the condition of exclusion for one block and adsorption for the other block was studied by Gorbunov and Vakhruskev in detail [17]. Theoretical calculations were performed by Jiang et al. using lattice Monte Carlo simulations with self-avoiding-walk chains to examine the effect of invisible blocks on the retention behavior of block copolymers in LCCC [18]. The simulated results are in good agreement with the experimental data and previous observed effects in the group of Chang in 2001 and 2003 could be confirmed to be of thermodynamic origin [19,20]. Wang et al. expanded the theoretical calculations for star branched polymers applying random-walk and self-avoiding-walk chain models [21]. A similar Monte Carlo study was performed by Ziebarth et al. about the adsorption and partitioning of the chains into pores using bulk Θ solutions, in which polymeric coils act like ideal polymer chains [22,23]. The thermodynamic background about the interaction of a polymer chain in a solution at the critical or Θ point is explained by Flory [24]. The main result is related to the information that the usage of simulations with self-avoiding-walks of the polymer chains do not coincide with previously performed calculations by Gorbunov and Vakhruskev, who applied the Gaussian chain theory [17]. Beside the theoretical calculation process, Schoenmakers et al. developed a design protocol for comprehensive two-dimensional liquid chromatography. Following this code of practice, suitable chromatographic parameters, such as column dimensions, flow rate and so on, can be selected effectively [25]. Additionally, Vivó-Truyols and Schoenmakers employed chemometric methods as useful tool to interpret and analyze two-dimensional chromatograms and to distinguish between different families of compounds belonging to one polymer class [26].

As second dimension an independent chromatographic technique or mass spectrometry technique has to be applied to gain a sufficient two-dimensional separation according to chemical composition or functionality on the first dimension and molar mass

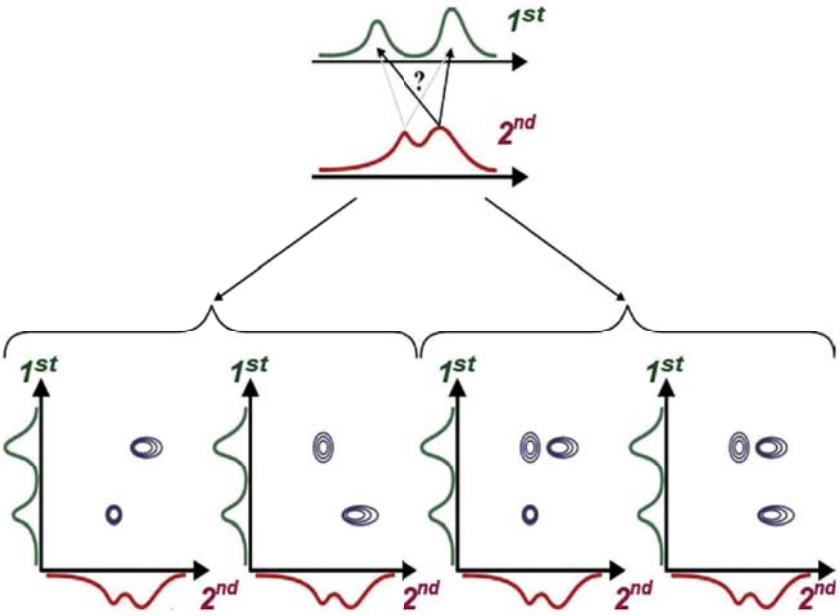

Fig. 1. Diagram for possible correlations of two chromatographic traces.

Reproduced from Ref. [15].

on the second dimension. As previously mentioned, SEC can be used as second chromatographic technique in an on-line approach. For this purpose, not only the LCCC part has to be optimized but also the SEC part. Adler and Kilz described the improvement of a 2D measurement by adjusting not only the separation setup but also the enhancement of the chromatographic data system using overlapping injection and a data reduction tool implemented in the 2D analysis software [15]. To achieve the best separation behavior for the chosen column polymer system, the construction of Poppe plots [27] and integrity plots [28] are useful, which depend on the column itself as well as the used eluent polymer system. Based on experimental data obtained for poly(styrene) (PS), Popovici et al. applied those Poppe and integrity plots for the consideration of the "right" SEC system [29,30]. The authors could show that the optimum column length for fast SEC measurements is greater than the 50 mm columns typically used. In addition, this research group studied also the band broadening in SEC to distinguish between chromatographic band broadening (due to the used column) and SEC selectivity (due to the sample polydispersity) by the usage of SEC × SEC analysis for a series of PS homopolymers [31]. Further investigations to avoid breakthrough of the weaker solvent or an adsorption phenomena in the second dimension were performed by Reingruber et al. [32,33]. The authors could clearly show that the optimization of the method and a solvent mixing step directly after the autosampler can avoid adsorption and breakthrough effects. Another possibility to achieve a faster separation in the 2nd dimension without much loss in resolution is the usage of high temperature-SEC (HT-SEC), whereby the mobile phase viscosity decreases and the diffusivity of the analytes increases [34,35]. For the optimization of on-line two-dimensional chromatography measurements Vivó-Truyols et al. developed a Pareto-optimality method to overcome the loss in peak capacity and the band broadening effect [36]. Applying a Pareto analysis yields optimal values for the measurement parameters to achieve the best relation between peak capacity, dilution and time.

2. Liquid chromatography investigations for synthetic polymers and biopolymers

Synthetic polymers as well as biopolymers can be very complex systems regarding their variation in chain length, chemical composition, functionality type as well as architecture. In addition, the

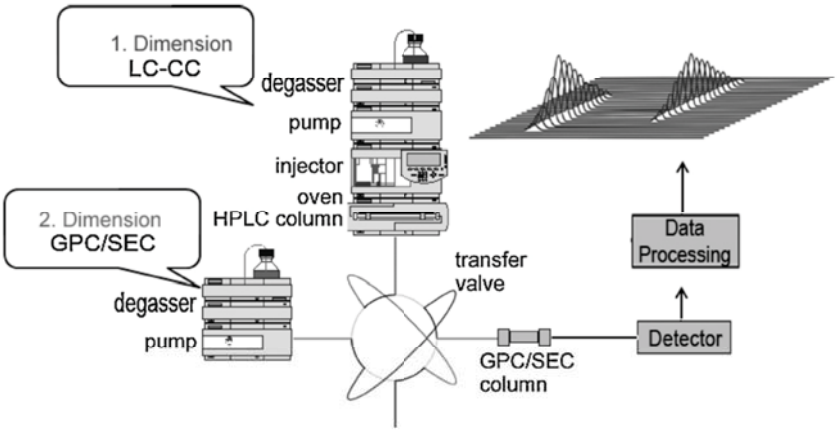

Fig. 2. Schematic representation of the 2D chromatography setup combining LCCC and SEC.

Reproduced from Ref. [184].

large variety of monomeric units in synthetic polymers is the main reason which is responsible for the complexity of the (co)polymer samples due to the existence of different dispersities. For a detailed description of heterogeneous synthetic polymer systems different chromatographic and combined techniques have to be utilized to analyze these macromolecules in detail. Pasch et al. summarized the advantages of hyphenated techniques in liquid chromatography for polymers in 2000 [37]. An overview about the investigations for synthetic polymers applying different liquid chromatography methods since 2005 is one of the topics in this section.

In contrast to synthetic polymers, biopolymers are defined as macromolecules assembled by living organisms, which are the carriers of the most important functions of living cells. Due to their natural character and their biodegradability or biocompatibility, they are of great interest for pharmaceutical and biomedical applications. In addition, they can be utilized as packing materials for food or as raw material in the areas of agriculture, horticulture and hygienic articles to produce foils, twines, screws or bone filling material. Beside proteins and nucleic acids, which are already discussed in several research articles in detail, the investigations for biopolymers are summarized as well in this section.

2.1. Liquid chromatography at the critical conditions (LCCC)

Liquid chromatography at the critical conditions represents a very powerful characterization tool for different synthetic polymers, like homopolymers, oligomers with different functional groups, polymer blends as well as block- and statistical copolymers. First steps towards the determination of the chemical composition dispersity (CCD) or the functionality type dispersity (FTD) of polymers were already performed by Tompa [38] and Flory [24]. Based on the studies about phase relationships in polymer solutions and the Flory-Huggins theory, which describes the limited solubility of polymers in solution, the term "critical point" was used probably for the first time. A comprehensive overview about the idea, which was already developed in the 1970s by Belenky et al. [39,40] and later supported by theoretical background studies by Skvortsov and Gorbunov [41,42], was given by Berek [10]. The basic concept behind this project was to develop a method for the complete separation of a copolymer and its corresponding homopolymer amounts or the separation of polymer analogous series according to their functionality type. The key role is represented by the interaction parameter c, which describes the interaction of a structural unit with the stationary phase in this chromatographic separation process [43]. In the ideal case, also termed as critical point of adsorption, this parameter equals zero applying a special mobile phase composition at a certain temperature on the used column for each polymer type. At this point the entropic and enthalpic effects compensate each other and all polymer chains of the same monomeric composition elute simultaneously independent on the molar mass, which means they become chromatographically "invisible" at the transition point [44]. By changing the ideal mobile phase mixture, the interaction parameter can be either negative (size-exclusion chromatography (SEC mode)) or positive (liquid adsorption chromatography (LAC mode)). In the SEC mode the retention time decreases with an increase in molar mass, whereby the entropy change is the driving force for the interaction of the polymer chains with the stationary phase [45]. In order to minimize interactions with the column material in a SEC measurement, a good solvent for the polymer as eluent helps to suppress these effectively [46]. On the other hand, the enthalpic interaction with the column material is the main factor for the LAC mode, where the retention time increases exponentially with an increase of the molar mass. A schematic representation of the three different interaction modes is given in Fig. 3.

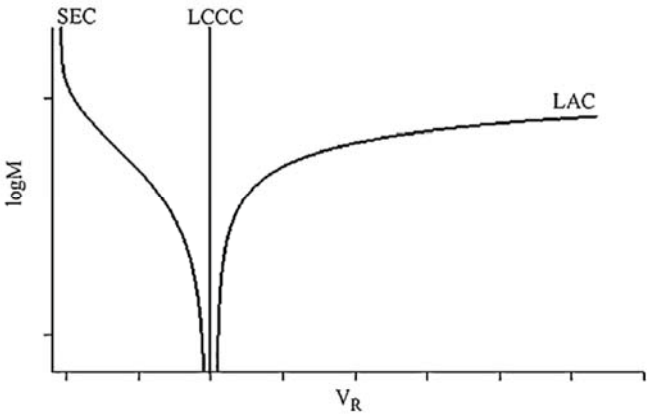

Fig. 3. Schematic representation of the dependences of elution volume on molar mass for size exclusion, adsorption and chromatography at critical conditions. Reproduced from Ref. [56].

As already mentioned before, the critical conditions can be reached using special settings. Therefore, the search for the transition point of a given polymer in HPLC includes the adjustment of several experimental parameters, such as the correct column material, the flow rate, the "special" solvent combination, the accurate temperature settings for the evaporative light scattering detector (ELSD) regarding the investigate solvent combination as well as the column oven temperature [25]. If these parameters are not carefully fitted to the system, reduced or low sample recovery can occur as major drawback. This behavior was studied in detail based on the pore size, the column material and different solvent combinations by Berek and coworkers [47] as well as by Beaudoin et al. [48,49] for poly(methyl methacrylate)s (PMMA) and PS as examples. Detailed investigations about the influence of the temperature and the pore size on the critical conditions were performed previously by Macko et al. [50] and later by Abdulahad and Ryu [51]. Using a fixed mobile phase composition, at larger pores the critical point of adsorption occurs at higher temperatures, which is equivalent to a weaker surface interaction energy. Due to slight variations of the column temperatures, it was possible to reach the critical point of adsorption more precisely by applying only pure solvents as mobile phases [52,53]. Besides these varieties, Brun and coworkers and later Bashir et al. applied this gradient chromatography technique to determine the critical eluent composition in a fast way for several polymer classes, such as poly(acrylate)s or poly(ether)s [54]. Additionally, Bashir and Radke summarized different models, such as the linear solvent strength model (LSSM), the quadratic solvent strength model (QSSM) as well as the polymer model (PM) to predict the retention times of several polymer classes (poly(ethylene oxide) (PEO), PS and PMMA) in isocratic and gradient chromatography without an extensive laboratory effort [55,56]. The authors showed that the classical models, LSSM and QSSM, represent a good projection of gradient retention times, which are based on gradient experiments. Unfortunately, these models fail for the critical point of adsorption in contrast to the application of the PM model. This model predicts the correct retention times requiring the use of suitable initial experiments for non-functionalized homopolymers under gradient as well as isocratic elution conditions. Further investigations in this research field were carried out by Trathnigg et al. developing a database for polymer chromatography for the prediction of the retention behavior based on the dependence of the interaction parameter on the mobile phase composition [57,58]. Gorbunov et al. extended the previous studies on the one hand to a quantitatively prediction model for the measurements of homopolymers and on the other hand to describe and simulate the theoretical retention behavior

of ring-shaped block copolymers in contrast to ideal linear ones [59–61]. But nevertheless, as major drawback should be mentioned that LCCC is only working for two kinds of macromolecules with distinct nature. Synthetic polymers consisting of three different components cannot be separated by this advanced characterization technique.

Based on all the gained knowledge about the determination of the critical point of adsorption (CAP), LCCC was already performed for several homo- and copolymer systems. An overview of the critical conditions for the different polymer classes is given in Tables 1–4 for the desired time range starting from 2005 similar to a previous summary given by Macko and Hunkeler in 2003 [62]. In these publications poly(alkylene oxide)s are the most studied polymer class up to now (see Table 1(A–C)). PEO represents a versatile material for industrial and medicinal applications and was investigated mainly on different reversed phase or polar columns [63]. An overview about the investigations for PEO using LC, in particular LCCC, and combined analysis techniques is given by Plata et al. [64]. The critical point of adsorption for this polymer type was found applying a methanol/water or acetone/water solvent combination. Due to the different end groups the transition point is slightly shifting. Therefore, the solvent composition has to be adjusted for all end group possibilities. As example, the solvent mixture, which is often used for poly(ethylene oxide) methyl ether, is around 80 vol% of methanol and 20 vol% of water on a reversed phase column and 78 vol% acetone mixed with 22 vol% water on a polar column [43,45,65]. In comparison to PEO, the more hydrophobic poly(propylene oxide) (PPO) homopolymers require a less polar solvent combination to reach the critical point of adsorption, for example a tetrahydrofuran (THF)/water mixture at around 80–85/20–15 (v/v) [66–68] or an acetone/water mixture at around 92/8 (v/v) or 45/55 (v/v) [44,65] used on different reversed phase columns, like HS-PEO or C18 packed columns provided by a range of companies. The identification of the CAP for higher poly(alkylene oxide)s, such as poly(butylene oxide) (PBO) or poly(hexylene oxide) (PHO), was performed on reversed phase columns, mainly C18 columns. In both cases, the mobile phase combination of THF/water led to overlapping of all polymer chains of PBO (around 85/15, v/v) [44,65,69] or PHO (around 90/10, v/v) [44,65,69] independent on the molar mass. Comprehensive thermodynamic studies about PEO and other poly(oxy ethylene)s were performed by Trathnigg et al. to utilize the CAP for non-ionic surfactants and emulsifiers [70,71]. Beside the column material and the mobile phase also the temperature was varied between 15.0 and 35.0 °C applying afterwards the best parameters from the specific van't Hoff plots for the separation of PEO polymers with varying end groups. LCCC as separation tool was also used for amphiphilic block copolymers consisting of PEO and PPO units on different HPLC columns for PEO and PPO [43,65]. With the application of the CAP for PEO it was possible to separate PEO-*b*-PPO diblocks from PEO-*b*-PPO-*b*-PEO triblocks using a solvent composition of methanol/water [43]. A continuative study was performed for self-synthesized PEO-*b*-PPO diblock copolymers in 2007 by the same group utilizing the critical conditions for the PPO block unit [68]. In 2009 Malik et al. expanded the study about amphiphilic block copolymers interpreting poly(ethylene oxide-*b*-ε-caprolactone) (PEO-*b*-PCL) [45] as well as PEO-*b*-PPO samples [72]. Based on their knowledge about the CAP for PEO and PPO the separation of these copolymers was performed using the respective critical conditions. In the study of Ahmed et al. it was possible to separate PEO-*b*-PCL polymers containing one to nine caprolactone units applying an acetonitrile (CH_3CN)/water mixture (69/31, v/v) for PEO as LCCC mobile phase composition [46]. Additionally, liquid exclusion adsorption chromatography (LEAC) was utilized as second characterization method to separate the different oligomers of a PEO-*b*-PCL block copolymer included in the LCCC fractions. Furthermore, Malik et al. focused on the characterization of amphiphilic block copolymers with higher alkylene oxides, *i.e.* PBO and PHO [44]. After evaluation of the critical conditions for PBO and PHO on a reversed phase column, the separation of the diblock copolymers (PEO-*b*-PBO and PEO-*b*-PHO) as well as the tri- and tetrablock copolymers (PEO-*b*-PPO-*b*-PBO or PEO-*b*-PPO-*b*-PBO-*b*-PHO) was performed successfully. Later on the authors used LCCC of PPO and higher alkylene oxides as characterization tool for the verification of pure homo- and copolymer products synthesized by microwave-assisted polymerizations [73,74].

The second major polymer class mentioned in Table 2(A–C) are the poly(acrylate)s, including various alkyl acrylates as well as methacrylate (MA) and methyl methacrylate (MMA). The critical conditions for PMA could be identified on a reversed phase column applying methylethylketone mixed with cyclohexane as mobile phase components [75]. In contrast, a normal phase column and a mobile phase consisting of CH_3CN and methylene chloride has to be used in order to reach the CAP for PMMA [12,76] or poly(butyl acrylate) (PBA) [76]. Applying critical conditions for PBA, Jacquin et al. could quantify the amount of unreacted PBA homopolymer after the synthesis of different poly(acrylate) block copolymers *via* controlled radical polymerization procedures [77,78]. To achieve an idea about the block-length dispersity in block methacrylate copolymers by comprehensive 2D chromatography, van Hulst et al. determined the critical behavior for PMMA as well as poly(butyl methacrylate) (PBMA) on different normal-phase and reversed-phase systems [79]. Critical conditions for PMMA could also be reached by variation of the column temperature in pure ethyl acetate. Hiller et al. used this different approach to separate poly(isoprene-*b*-methyl methacrylate) (PI-*b*-PMMA) block copolymers from parent homopolymers and, furthermore, hyphenation with nulcear magnetic resonance (^{1}H NMR) spectroscopy delivered the correct chemical composition of the block copolymer [53]. Two-dimensional chromatography was applied to the mentioned poly(acrylate) studies and is explained in detail later.

As third main polymer group, PS has to be noted (see Table 3). The identification of the CAP for this polymer class can be either performed by the conventional method using a non-solvent/solvent combination, such as THF/cyclohexane [80], methylene chloride/CH_3CN [51,81,82] and THF/*n*-hexane [83,84], or a pure solvent by tuning the temperature [52]. In addition, a mobile phase combination of *iso*-octane/THF in combination with a temperature gradient program resulted in a good separation of PS-*b*-PI samples close to the critical conditions of PS [85]. Cho et al. investigated critical conditions for PS to separate cyclic polystyrenes from linear chains [86]. Galindo et al. used this technique to separate non-grafted PS from poly(butadiene)-*g*-poly(styrene) (PB-*g*-PS) and ungrafted PB as well as to determine the grafting yield by single UV detection [83]. Further investigations of PS containing block copolymers were performed by Malik et al. determining the block length of different poly(dimethylsiloxane-*b*-styrene) (PDMS-*b*-PS) copolymers and blends [87].

In addition, LCCC was also implemented to other polymer classes, such as poly(2-ethyl-2-oxazoline) (PEtOx), poly(carbonate) (PC), PCL, as well as other synthetic polymers using optimized measurement conditions. The applied parameters and settings are summarized together with those of the biopolymers in Table 4 and continuative two-dimensional measurements are mentioned afterwards in the corresponding paragraphs.

Nevertheless, the extensive search for the critical point of adsorption was also performed for some biodegradable polymers, such as adipinic acid, poly(lactide) or PI. PI represents the most studied polymer type in this field. The critical conditions can be reached either on normal phase columns or reversed phase columns using a solvent-combination of THF and *iso*-octane [82]

Table 1
Liquid chromatography conditions for different poly(oxy ethylene)s: isocratic mode: (A) poly(ethylene oxide); (B) poly(propylene oxide); (C) higher poly(oxy ethylene)s.

Endgroups	HPLC solvent mixture	Column	Flow rate
(A) Poly(ethylene oxide)			
$H(CH_2)_{18}[O—CH_2—CH_2]_nOH$	CC: 15.7/84.3 (v/v) (A/B) [93] (A) H_2O 0.1% formic acid (B) CH_3CN/MeOH 30/70 (v/v)	Waters BEH C18 (50 mm × 2.1 mm) particle size: 1.7 μm oven temperature: 30 °C	0.35 mL/min
$CH_3[O—CH_2—CH_2]_nOH$	CC: 78/22 (v/v) (acetone/H_2O) [43–45,65]	Macherey-Nagel Nucleosil 100-5 OH 5 μm (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
$CH_3[O—CH_2—CH_2]_nOH$	CC: 69/31 (v/v) (CH_3CN/H_2O) [46]	PhaseSep Spherisorb ODS2 (300 mm × 25 mm) particle size: 5 μm oven temperature: 25 °C	2.0 mL/min
$H[O—CH_2—CH_2]_nOH$	CC: 81/19 (v/v) (MeOH/H_2O) [94]	Knauer Nucleosil RP18-100	
$H[O—CH_2—CH_2]_nOH$	CC: 82.5/17.5 (v/v) (MeOH/H_2O) [94]	Nucleosil C_{18}	
$CH_3[O—CH_2—CH_2]_nOH$	CC: 81/19 (v/v) (MeOH/H_2O) [95]	Knauer Nucleosil RP18-100 (250 mm × 4.6 mm)	
$CH_3[O—CH_2—CH_2]_nOH$	CC: 75.7/24.3 (v/v) (acetone/H_2O) [69]	Macherey-Nagel Nucleosil 100-5 OH 5 μm (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
$CH_3[O—CH_2—CH_2]_nOH$	CC: 90/10 (v/v) and 25–30/75–70 (v/v) (acetone/H_2O) or 80/20 (v/v) (MeOH/H_2O) [43]	Waters Novapak C18 (300 mm × 3.9 mm) Particle size: 4 μm oven temperature: 25 °C	0.5 mL/min
$CH_3[O—CH_2—CH_2]_nOH$	CC: 30/70 (v/v) (acetone/H_2O) [43]	Jordi Gel DVB 500 RP (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
$CH_3[O—CH_2—CH_2]_nOH$	CC: 88.8/11.2 (v/v) (MeOH/H_2O containing 1 mM LiCl) [96–98]	Cluzeau Equisil C18 (150 mm × 4 mm) particle size: 5 μm oven temperature: 25 °C	1 mL/min
$CH_3[O—CH_2—CH_2]_nOH$	CC: 93.5/6.5 (v/v) (MeOH/H_2O containing 1 mM LiCl) [98]	Cluzeau Nucleosil (250 mm × 4 mm) particle size: 5 μm oven temperature: 25 °C	1 mL/min
$CH_3[O—CH_2—CH_2]_nOH$	CC: 80/20 (v/v) (MeOH/H_2O) [66]	ODS columns	
$CH_3[O—CH_2—CH_2]_nOH$	CC: 70–95/30–5 (v/v) (MeOH/H_2O) [66]	C18 columns	
$C_4H_9[O—CH_2—CH_2]_nOH$	CC: 84/16 (v/v) (MeOH/H_2O) [67]	Two YMC RP 18 columns (150 + 150 mm × 4.6 mm) particle size: 5 μm oven temperature: 45 °C	0.5 mL/min
$H[O—CH_2—CH_2]_nOH$	CC: 84/16 (v/v) (MeOH/H_2O) [99,100]	Two YMC C18 columns (250 + 150 mm × 4.6 mm) oven temperature: 45 °C	
$[O—CH_2—CH_2]_n$	CC: 85.8/14.2 (v/v) (MeOH/H_2O) [71]	Zorbax 300 C18 (150 mm × 4.6 mm) particle size: 3.5 μm	0.5 mL/min
$[O—CH_2—CH_2]_n$	CC: 89.6/10.4 (v/v) (acetone/H_2O) [71]	Prodigy ODS3 (250 mm × 4.6 mm) particle size: 5 μm	0.5 mL/min
$[O—CH_2—CH_2]_n$	CC: 90/10 (v/v) (acetone/H_2O) [101]	Phenomenex Onyx C18 (100 mm × 4.6 mm)	2 mL/min
$[O—CH_2—CH_2]_n$	CC: 32/68 (v/v) (acetone/H_2O) [102]	Phenomenex Synergi Fusion RP (250 mm × 4.6 mm) Particle size: 4 μm	0.5 mL/min
$[O—CH_2—CH_2]_n$	CC: 83/17 (v/v) (MeOH/H_2O) [54]	Macherey-Nagel Nucleosil C18 (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 35 °C	1 mL/min
(B) Poly(propylene oxide)			
Purchased by PSS	CC: 15/85 (v/v) (A/B) [93] (A) H_2O 0.2% formic acid (B) THF	Waters BEH C18 (50 mm × 2.1 mm) particle size: 1.7 μm oven temperature: 30 °C	0.2–0.5 mL/min
$H[O—CH—CH_3—CH_2]_nOH$	CC: 92.42/7.58 (v/v) (acetone/H_2O) [43,44,65] or 80.22/19.78 (v/v) (THF/H_2O) [44,45,68,73]	Jordi Gel DVB 500 RP (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
$H[O—CH—CH_3—CH_2]_nOH$	CC: 45.58/54.42 (v/v) (acetone/H_2O) [65]	Discovery HS-PEG (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
$H[O—CH—CH_3—CH_2]_nOH$	CC: 85/15 (v/v) (THF/H_2O) [66]	Phenomenex Prodigy ODS3 silica-based octadecyl phase (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	2.0 mL/min
$H[O—CH—CH_3—CH_2]_nOH$	CC: 81.14/18.86 (v/v) (THF/H_2O) [66]	PhaseSep Spherisorb ODS2 (300 mm × 25 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
$H[O—CH—CH_3—CH_2]_nOH$	CC: 85/15 (v/v) (THF/H_2O) [67]	Two YMC RP 18 columns (150 + 150 mm × 4.6 mm) particle size: 5 μm oven temperature: 45 °C	0.5 mL/min
$H[O—CH—CH_3—CH_2]_nOH$	CC: 90/10 (v/v) (MeOH/H_2O) [45]	PhaseSep Spherisorb S5P (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
$[O—CH—CH_3—CH_2]_n$	CC: 87/13 (v/v) (CH_3CN/H_2O) [72]	Macherey-Nagel Nucleosil Si 120 (250 mm × 4 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min

Polymer	Endgroups	HPLC solvent mixture	Column	Flow rate
(C) Higher poly(oxy ethylene)s				
PBO	$H[C_4H_8O]_nOH$	CC: 84.55/15.45 (v/v) (THF/H_2O) [44,45,74]	Jordi Gel DVB 500 RP (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
	$H[C_4H_8O]_nOH$	CC: 84.55/15.45 (v/v) (THF/H_2O) [45]	Prodigy ODS3 Phenomenex (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
	$[C_4H_8O]_n$	CC: 88/12 (v/v) (THF/H_2O) [69]	PhaseSep Spherisorb ODS2 (250 mm × 10 mm) particle size: 5 μm oven temperature: 25 °C	2.0 mL/min
PHO	$H[C_6H_{12}O]_nOH$	CC: 87.04/12.96 (v/v) (THF/H_2O) [44,45,74]	Jordi Gel DVB 500 RP (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
	$[C_6H_{12}O]_n$	CC: 92/8 (v/v) (THF/H_2O) [69]	PhaseSep Spherisorb ODS2 (250 mm × 10 mm) particle size: 5 μm oven temperature: 25 °C	2.0 mL/min

or pure dioxane [53] at a certain temperature. Radke et al. identified the critical point of adsorption for linear poly(lactide)s at 50 °C with 1,4-dioxane/*n*-hexane as mobile phase eluent and applied it to functionalized star-shaped polymers [88]. Using a Gaussian chain model for large and small pores, theoretical calculations of the retention behavior led to equally good descriptions of the experimental data. In addition, a first step towards applying the critical conditions in a faster way also for other biopolymer types was demonstrated by Gorshkov et al. in 2006. The authors developed a sequence-dependent retention time prediction tool based on the concept of LCCC for peptides and biopolymers, the BioLCCC [89,90]. Theoretical calculations of the model are based on

Table 2
Liquid chromatography conditions for different poly(acrylate)s: isocratic mode: (A) poly(alkyl acrylate)s; (B) poly(methacrylate)s; (C) poly(methyl methacrylate).

Polymer	Endgroups	HPLC solvent mixture	Column	Flow rate
(A) Poly(acrylate)s				
PiBoA	$C_7H_7(C_{13}H_{20}O_2)_nC_6H_4NS_2$	CC: 68/32 (v/v) (THF/MeOH) [103,104]	Zorbax Eclipse XDB-C18 columns (150 mm × 4.6 mm) particle size: 5 μm oven temperature: 35 °C	0.08 mL/min
PiBA		CC: 6/94 (v/v) (CH_3CN/CH_2Cl_2 spiked with acetic acid) [76]	Hypersil Silica (150 mm × 4.6 mm) particle size: 3 μm oven temperature: 25 °C	0.5 mL/min
(B) Poly(meth acrylate)s				
PBMA	$[C_8H_{14}O_2]_n$	CC: 75/25 (v/v) (THF/*n*-heptane) [79]	Macherey-Nagel Nucleosil (250 mm × 4.6 mm) particle size: 7 μm oven temperature: 25 °C	1 mL/min
PMA	$C_6H_{11}O_2$ $[CH_2CHCOOCH_3]_nBr$	CC: 86/14 (v/v) (MEK/cyclohexane) [75]	Macherey-Nagel Nucleosil 100-5 and 300-5 (250 mm × 4 mm) particle size: 5 μm oven temperature: 32 °C	0.5 mL/min
PBA	$[C_7H_{11}O_2]_n$	CC: 90/10 (v/v) (THF/H_2O) [77,78]	Zorbax SB C18 (250 mm × 4.6 mm) room temperature	1 mL/min

Endgroups	HPLC solvent mixture	Column	Flow rate
(C): poly(methyl methacrylate)			
$HO[CH_2CCH_3COOCH_3]_nOH$	CC: difunctionalized PMMA: 48/52 (v/v) (CH_3CN/CH_2Cl_2) monofunctionalized PMMA: 52/48 (v/v) (CH_3CN/CH_2Cl_2) nonfunctionalized PMMA: 56/44 (v/v) (CH_3CN/CH_2Cl_2) [12]	ThermoQuest two Hypersil "bare" silica columns (150 mm × 1.0 mm) particle size: 3 μm room temperature	8 μL/min
$[CH_2CCH_3COOCH_3]_n$	CC: 42/58 (v/v) (CH_3CN/CH_2Cl_2) [76]	Hypersil Silica (150 mm × 4.6 mm) particle size: 3 μm oven temperature: 25 °C	0.5 mL/min
$[CH_2CCH_3COOCH_3]_n$	CC: 23.5/66.5 (v/v) (*n*-hexane/THF) [79]	Hypersil Silica (150 mm × 4.6 mm) particle size: 3 μm oven temperature: 25 °C	1 mL/min
$[CH_2CCH_3COOCH_3]_n$	CC: 55/45 (v/v) (THF/CH_3CN) [79]	Discovery C18 (150 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.1 mL/min
$[CH_2CCH_3COOCH_3]_n$	CC: 54/46 (v/v) (CH_2Cl_2/CH_3CN) [105]	Nucleosil C18 oven temperature: 40 °C	1.8 mL/min
$[CH_2CCH_3COOCH_3]_n$	CC: pure ethyl acetate [53]	Two Macherey-Nagel Nucleosil Si 300-5 and 1000-5 (200 mm × 4.6 mm) Oven temperature: 10 °C	0.5 mL/min
$[CH_2CCH_3COOCH_3]_n$	CC: 37/63 (v/v) (THF/toluene) [54]	Macherey-Nagel Nucleosil bare silica (250 mm × 4 mm) Particle size: 7 μm Oven temperature: 35 °C	1 mL/min
$[CH_2CCH_3COOCH_3]_n$	CC: 74.3/25.7 (v/v) (MEK/cyclohexane) [106]	Macherey-Nagel Nucleosil Si 300-5 and 1000-7 (250 mm × 4 mm) particle size: 5 μm and 7 μm oven temperature: 22 °C	0.5 mL/min

different notions, *e.g.* a random walk model for a macromolecule chain. This calculation tool was later applied and extended to predict retention times also for the LC–MS phosphopeptide analysis as well as the accurate assignment of the amino acid sequence in biomacromolecules [91,92].

2.2. Liquid chromatography in the gradient mode

Another possibility to achieve a good separation of different polymeric systems in a HPLC measurement is the application of HPLC in the gradient mode, which has been described as gradient polymer elution chromatography (GPEC) by Cools [124,125]. This concept can be used to separate a wide range of homo- or copolymeric species as well as to monitor the amount of polymer in pure monomer in a fast way without the immense effort to identify the critical point of adsorption. Applying this separation procedure, the macromolecules stay in the beginning of the separation at the column entrance and flushed consequently through the column due to increasing the mobile phase component, which promotes polymer elution. A summary of the investigated synthetic polymers as well as biopolymers and the corresponding chromatographic parameters are represented in Table 5.

In the field of synthetic polymers only a small number of publications investigated HPLC gradient measurements in the chosen time range. In order to achieve the CAP for the different poly(alkyl acrylate)s, gradient HPLC measurements were performed to separate the homopolymers of each other using a THF/CH_3CN or THF/water mobile phase [126,127]. Takahashi et al. separated statistical copolymers consisting of styrene, MMA, BMA and 2,2,3,3,4,4,4-heptafluorobutyl methacrylate (FBMA) with liquefied CO_2 as one part of the mobile phase and a mixture of $CHCl_3$/EtOH in the gradient mode [128]. For the separation of linear and star-shaped PS samples Gerber and Radke utilized first HPLC measurements in the gradient mode using a mixture of THF and CH_3CN to achieve an appropriate separation method for further two-dimensional measurements [129,130]. Gradient measurements were also performed for various PCL homo- and copolymers using aqueous mobile phases [116,131,132]. Simple reversed phase LC (RPLC) measurements in the gradient mode were investigated for PPO by Jandera et al. [133,134]. Applying gradient elution along a critical adsorption line, Trathnigg et al. showed that the separation of PEO-*b*-PPO block copolymers could be also performed using RP columns in either a binary or a ternary mobile phase consisting of acetone, methanol and water [135]. In a second publication about the critical adsorption line for poly(ether)s, Cuong and Trathnigg published a method to determine the interaction parameters in ternary mobile phases by linear interpolation [136]. Poly(olefin)s have become the most important synthetic polymers in the last years. Due to its semicristalline structure, which requires characterization temperatures above 100 °C, SEC was mainly investigated as characterization technique. Starting from 2005 also first tremendous HPLC investigations in the gradient mode were performed at higher temperatures of 140 up to 160 °C using a solvent combination of trichlorobenzene with either decanol[137–144], decane [145], cyclohexanone [146,147], or ehtylene glycol monobutyl ether[148–151] as well as a mixture of decaline with cyclohexanone [152,153]. Furthermore, Chitta et al. performed a complete screening for the separation of poly(olefin)s by changing the mobile as well as the stationary phase [154]. An important step to separate various generations of poly(amidoamine) (PAMAM) dendrimers was the usage of gradient HPLC measurements applying a linear gradient of the mobile phase from water to CH_3CN [155,156]. Poly(ester)s consisting of adipate and terephthalate units can be analyzed with a mobile phase mixture of THF and water [157].

Most of the studies dealing with biopolymer samples apply liquid chromatography in the gradient mode to achieve a good

Table 3
Liquid chromatography conditions for poly(styrene)s: isocratic mode.

Poly(styrene)s				
Polymer	Endgroups	HPLC solvent mixture	Column	Flow rate
PS	$C_4H_9[C_8H_8]_nOH$	CC: 18/82 (v/v) (THF/cyclohexane) [80]	Macherey-Nagel Nucleosil (300 + 250 mm × 4.6 mm) particle size: 5 μm	
	$C_6H_5[C_8H_8]_nOH$	CC: 88.4/11.6 (v/v) (THF/H_2O) [107]	YMC-ODSA RP 18 (250 mm × 3 mm) particle size: 5 μm oven temperature: 40 °C	0.5 mL/min
	$[C_8H_8]_n$	CC: pure DMF [52]	Macherey-Nagel Nucleodur Gravity C18 (250 mm × 4.6 mm) particle size: 3 or 5 μm oven temperature: 72 °C	0.08 mL/min
	$C_4H_9[C_8H_8]_n[C_8H_8]_n$ $SiCl_3CH_3$ (branched PS)	CC: 53/47 (v/v) (CH_2Cl_2/CH_3CN) [81]	Kromasil C18 (150 mm x 4.6 mm) oven temperature: 53.3 °C	0.5 mL/min
	$[C_8H_8]_n$	CC: 49/51 or 48/52 (v/v) (THF/CH_3CN) [108,109]	Macherey-Nagel Nucleosil 300-5 C18 and Nucleosil 1000-7 C18 (each 250 mm × 4 mm) particle size: 5 and 7 μm oven temperature: 32 °C or 22 °C	0.5 mL/min
	$[C_8H_8]_n$	CC: 52.4/47.6 (v/v) (THF/n-hexane) [48,83]	Two Macherey-Nagel Nucleosil 300-7 and 1000-7 (250 mm × 4.6 mm) particle size: 7 μm oven temperature: 30 °C	1 mL/min
	$[C_8H_8]_n$	CC: 43/57 (v/v) (THF/hexane) [84]	Two YMC columns 8 (250 mm × 4 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
	$[C_8H_8]_n$	CC: 58.1/41.9 (v/v) (CH_2Cl_2/n-hexane) [48]	Two Macherey-Nagel Nucleosil 300-7 and 1000-7 (250 mm × 4.6 mm) particle size: 7 μm oven temperature: 30 °C	1 mL/min
	$[C_8H_8]_n$	CC: 57/43 (v/v) (CH_2Cl_2/CH_3CN) [51]	Macherey-Nagel Nucleosil (250 mm × 4.6 mm) Particle size: 5 μm Oven temperature: 25–30 °C (depends on pore size)	0.3 mL/min
	$[C_8H_8]_n$	CC: 80/20 (v/v) (CH_2Cl_2/CH_3CN) [82]	Nucleosil C18 oven temperature: 20 °C	1.5 mL/min
	$[C_8H_8]_n$	CC: 35/65 (v/v) (iso-octane/THF) [105]	Nucleosil bare silica column oven temperature: 40 °C	1.8 mL/min
	$[C_8H_8]_n$	near CC: 97/3 (v/v) (*iso*-octane/THF) [85]	Nucleosil diol-bonded silica column (250 mm × 7.8 mm) particle size: 7 μm temperature gradient program	0.05 mL/min
	$[C_8H_8]_n$	CC: 55/45 (v/v) (*n*-hexane/THF) [87]	Macherey-Nagel Nucleosil Si 300 (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 30 °C	0.5 mL/min
	$[C_8H_8]_n$	CC: 57/43 (v/v) (CH_2Cl_2/CH_3CN) [110]	C18 ODS-80TsQA (250 mm × 8 mm) particle size: 5 μm oven temperature: 40.5 °C	0.5 mL/min
	$[C_8H_8]_n$	CC: 51.5/48.5 (v/v) (CH_3CN/THF) [79]	Supelco C18 (150 mm × 2.1 mm) particle size: 5 μm oven temperature: 25 °C	0.01 mL/min
	$[C_8H_8]_n$	CC: 57/43 (v/v) (CH_2Cl_2/CH_3CN) [86]	Hypersil C18 (150 mm × 4.6 mm) particle size: 3 μm oven temperature: 36.5 °C	0.5 mL/min

separation (see Table 5). Oligosaccharides can be clearly separated using a solvent gradient of ammonium formate solution at pH 4.4 in water and acetonitrile on normal phase columns [158,159]. In contrast to oligosaccharides, hydroxylpropylmethylcellulose (HPMC) requires a mobile phase consisting of a solvent combination of trifluoroacetic acid (TFA) in water and TFA in 1-propanol running in the gradient mode at low temperatures to avoid gelation of the polymer samples [160]. The separation of glycans can be performed on a hydrophilic interaction porous layer open tubular (HILIC-PLOT) column in gradient elution mode with a mixture of different amounts of water and CH_3CN spiked with formic acid [161]. For the determination of the total nondigestible carbohydrate amount in foods, official methods including LC separation are carried out using water as mobile phase at 80 °C for insoluble dietary fibers, nondigestible oligosaccharides as well as resistant starch [162]. Separating sulphonated lignins can be performed using an ion-pair agent as additive for the mobile phase mixture [163]. The application of gradient measurements for poly(vinyl acetate) (PVAc) homopolymers grafted onto cellulose derivatives was established by Fleet et al. using a mobile phase mixture of CH_3CN/water with 0.1% formic acid [164].

2.3. Liquid chromatography in the isocratic mode (LAC, LEAC, LCD)

Beside the LCCC or LC measurements in the gradient mode, also liquid chromatography in the isocratic mode is often applied as first dimension for further two-dimensional separations of polymers. A detailed explanation about the separation procedures can be found in the review given by Berek [10]. All details about the parameters used for the liquid chromatography measurements are summarized in Table 6.

One possibility to achieve a good separation according to the block length or chemical composition is the application of normal phase or reversed phase liquid chromatography (NPLC or RPLC) at an isocratic mobile phase composition. Using normal or reversed phase columns, the polymer samples elute mostly in the liquid adsorption mode based on the chosen mobile phase composition. Theoretical aspects about the parameters of liquid adsorption chromatography (LAC) and their prediction are summarized by Trathnigg et al. using PEO as polymeric example [166,167]. In the field of poly(alkylene oxide)s, Trathnigg et al. determined the accessible volume and the adsorption interaction parameter in the adsorption mode for PEO as well as PPO [168]. LAC measurements were also performed for different copolymers consisting of BA, MMA, acrylic acid and PS by Nicolas et al. [169] as well as Lefay et al. [170]. PI containing (co)polymers can be separated using a mixture of CH_2Cl_2/CH_3CN on NPLC columns [85].

Beside NPLC measurements, also the liquid exclusion adsorption chromatography (LEAC) represents an important tool for the separation of block copolymers in which one block is following the LAC mode, whereas the second block is excluded. Ahmed and Trathnigg studied in detail the characterization of PEO-*b*-PCL block copolymers using LEAC as second separation technique. The authors could clearly show that it was possible to distinguish between the diverse blocks according to the number of PCL repeating units [46]. Lacharme et al. extended previous measurements in this field and verified the theory of Trathnigg et al. [171,172] investigating a model diblock copolymer of alkyl phenol ethoxylates (APE) [173].

Table 4
Liquid chromatography conditions for other synthetic polymers and biopolymers: isocratic mode.

Other synthetic polymers				
Polymer	Endgroups	HPLC solvent mixture	Column	Flow rate
PC	$HO[C_{16}H_{14}O_3]_nH$	CC: 98.5/1.5 (v/v) ($CHCl_3$/Et_2O) [111,112]	Chrompack Inertsil platinum silica column (150 mm × 3.2 mm) particle size: 5 μm oven temperature: 30–33 °C	0.3 mL/min
	$HO[C_{16}H_{14}O_3]_nH$	CC: 98.5/1.5 (v/v) ($CHCl_3$/Et_2O) [113]	Alltech Platinum Silica column (150 mm × 3.2 mm) particle size: 5 μm oven temperature: 33 °C	0.3 mL/min
PCL	$[C_6H_{10}O_2]_n$	CC: 80/20 (v/v) (THF/H_2O) [46]	Reversed phase column	
	$CH_3[C_6H_{10}O_2]_nH$	CC: 81/19 (v/v) (THF/H_2O) [114]	Jordi Gel DVB 500 RP (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	0.5 mL/min
	$[C_6H_{10}O_2]_n$	CC: 82.5/17.5 (v/v) (THF/H_2O) [115]	Four Macherey-Nagel Nucleosil 120-5 C18 columns (250 mm × 4 mm) oven temperature: 40 °C	0.5 mL/min
	$[C_6H_{10}O_2]_n$	CC: 69/31 (v/v) (acetone/H_2O) [116]	PhaseSep Spherisorb ODS2 (25 x 300 mm) particle size: 5 μm oven temperature: 25 °C	2.0 mL/min
Epoxy resin	$C_{18}H_{19}O_2[C_{18}H_{20}O_3]_nC_3H_5O_2$	CC: 48–52/52–48 (v/v) (A/B) [117] (A) 1/99 (v/v) (MeOH/CH_2Cl_2) (B) 6/94 (v/v) (MeOH/CH_2Cl_2)	Agilent Rx-SIL (100 mm × 2.1 mm) particle size: 1.8 μm oven temperature: 30 °C	2.1 mL/min
PVP	$[C_6H_9NO]$	CC: 30/70 (v/v) (DMAc/CH_3CN) or 35/65 (v/v) (HFIP/CH_3CN) [118]	Discovery-Cyano (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 40 °C	0.5 mL/min
PEtOx	$CH_3[C_5H_9NO]_nOCOCH_3$	CC: 91/9 (v/v) (2-propanol/H_2O) [119]	Supelco Discovery CN column (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 35 °C	1 mL/min
PDMS	$[C_2H_6SiO]_n$	CC: 41/59 (v/v) (MeOH/THF) [87]	Phenomenex Jupiter Octadecyl (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 30 °C	0.5 mL/min
PVAc	$[CH_2CHOH]_n$ (Standard from PSS)	CC: 71.8/28.2 (v/v) (THF/H_2O) [120]	Macherey-Nagel Nucleodur C18 Pyramid (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 35 °C	1 mL/min
	$[CH_2CHOH]_n$	CC: 35/65 (v/v) (trichlorobenzene/cyclohexanone) [121]	Macherey-Nagel Nucleosil 500 (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 140 °C	1 mL/min
PEster		CC: 94/6 (v/v) (acetone/THF) [122]	Macherey-Nagel Nucleosil C18 (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 25 °C	1 mL/min
PI	$C_4H_9[C_5H_8]_nOH$	CC: 91.5/8.5 (v/v) (MEK/cyclohexane) [80]	Macherey-Nagel Nucleosil (300 + 250 mm × 4.6 mm) particle size: 5 μm	
	$[C_5H_8]_n$	CC: 75/25 (v/v) (*iso*-octane/THF) [82]	Nucleosil diol-bonded silica column oven temperature: 15 °C	1.5 mL/min
	$[C_5H_8]_n$	CC: pure dioxane [53]	Two Macherey-Nagel Nucleosil C18 300-5 and 1000-7 (250 mm × 4 mm) oven temperature: 54 °C	0.5 mL/min
PLA (linear)	$[C_3H_4O_2]_n$	CC: 57.1/42.9 (v/v) (1,4 dioxane/*n*-hexane) [88]	Two silica Nucleosil (200 + 250 mm × 4.6 mm) oven temperature: 50 °C	0.5 mL/min
Adipinic acid	$HO[CO(CH_2)_4COO]_nH$	CC: 48/52 (v/v) (acetone/*n*-hexane) [123]	Macherey-Nagel two silica gel columns (250 + 125 mm × 4.6 mm) particle size: 5 μm oven temperature: 45 °C	0.5 mL/min

The third often applied possibility to achieve a separation according to the chemical composition is liquid chromatography under limiting conditions of desorption (LC LCD) [174]. In this special technique, the LC eluent promotes desorption of the polymers. As a consequence, it is possible to separate both parent homopolymers in one single step from the corresponding copolymer. LC LCD was introduced by Berek et al. and already applied for different homo- and copolymer samples, such as PMMA [175–178], PS [176–178], PVAc [177], poly(ethylene terephthalate) (PET) [179], poly(butylenes terephthalate) (PBT) [179], poly(lactide) [179], and PS-*b*-PMMA [177,180,181]. The used parameters for the LC LCD measurements are summarized in Table 6.

3. Combined analysis techniques for synthetic polymers and biopolymers

The combination of different chromatographic separation techniques as well as the hyphenation of LC with mass spectrometry provides a perfect tool for the detailed analysis of the polymeric structure. The theoretical background of two-dimensional chromatography as characterization tool was already discussed in Section 1. The most important step for such an analysis of functionalized polymers is the usage of two independent chromatographic techniques for the separation according to the FTD and the molar-mass dispersity (MMD) [12]. Another possible combination of isolation techniques for (co)polymers is based on the separation according to the CCD and the MMD. The following sections will provide an overview about the different coupling possibilities and their application to polymers.

3.1. Liquid chromatography coupled with size exclusion chromatography (LC–SEC)

The combination of liquid chromatography as first separation technique, which can be employed either in the gradient or in the isocratic mode, and SEC as second characterization technique, represents the most common two-dimensional chromatography setup in the field of synthetic polymers. In addition, the application of the critical point of adsorption as first analysis step is often investigated as documented in a range of publications. Simulation of the separation behavior and the corresponding 2D contour plots for diblock copolymers were performed and compared to real 2D contour plots

Table 5
Liquid chromatography conditions applying gradient measurements.

Polymer	HPLC solvent mixture	Column	Flow rate
PiBoA	Gradient measurement: 58.5/41.5 (v/v) (THF/CH_3CN) [126]	Polymer Laboratories PLRP-S (150 mm × 4.6 mm) particle size: 5 μm	1 mL/min
PiBA	Gradient measurement: 33.2/66.8 (v/v) (THF/CH_3CN) [126]	Polymer Laboratories PLRP-S (150 mm × 4.6 mm) particle size: 5 μm	1 mL/min
PBA	Gradient measurement: (A) THF/H_2O (50/50 (v/v)) (B) THF [127]	Alltech Kromasil C18 (150 mm × 4.6 mm) oven temperature: 30 °C	
PEHA	Gradient measurement: 48.9/51.1 (v/v) (THF/CH_3CN) [126]	Polymer Laboratories PLRP-S (150 mm × 4.6 mm) particle size: 5 μm	1 mL/min
PiBMA	Gradient measurement: 37.8/62.2 (v/v) (THF/CH_3CN) [126]	Polymer Laboratories PLRP-S (150 mm × 4.6 mm) particle size: 5 μm	1 mL/min
PiBoMA	Gradient measurement 60.1/39.9 (v/v) (THF/CH_3CN) [126]	Polymer Laboratories PLRP-S (150 mm × 4.6 mm) particle size: 5 μm	1 mL/min
PS	Solvent gradient measurement: 35/65 → 51/49 (v/v) (THF/CH_3CN) Temperature gradient measurement: 50/50 or 52/48 (v/v) (THF/CH_3CN) [129,130]	Macherey-Nagel Nucleosil C18 (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 35 °C or gradient	0.2 mL/min
PCL	Gradient measurement: (A) 5 mM ammonium acetate (B) 90/10 (v/v) (CH_3CN/solution (A)) [131]	Xterra MS C8 (150 mm × 2.1 mm) particle size: 3.5 μm oven temperature: 20 °C	200 μL/min
	Gradient measurement: (A) 20 mM ammonium formate buffer (B) CH_3CN [132]	Macherey-Nagel C8 (150 mm × 2.1 mm)	300 μL/min
	Gradient measurement: (A) acetone (B) H_2O [116]	Phenomenex Synergi Fusion RP column (250 mm × 4.6 mm) particle size: 4 μm	0.5 mL/min
PEO	Gradient measurement: (A) acetone/H_2O (B) MeOH/H_2O [135,136]	Phenomenex Synergi MAX RP (250 mm × 4.6 mm) particle size: 4 μm	0.5 mL/min
PPO	Gradient measurement: (A) CH_3CN/H_2O (B) CH_3CN [133,134]	Agilent Zorbax 300 Extend C18 RP column (150 mm × 4.6 mm) particle size: 5 μm oven temperature: 40 °C	0.5 mL/min or 0.2 mL/min
PAMAM	Gradient measurement: (A) H_2O (B) CH_3CN [155,156]	Phenomenex Jupiter C5 RP column (250 mm × 4.6 mm) particle size: 5 μm	1 mL/min
Poly(olefin)s	Gradient measurement: (A) decane (B) trichlorobenzene [145]	Hypercarb porous graphite column (100 mm × 2.1 mm) particle size: 5 μm oven temperature: 140 °C	1 mL/min
	Gradient measurement: (A) decanol (B) trichlorobenzene [137–144]	Hypercarb column (250 or 100 mm × 4.6 mm) particle size: 5 μm oven temperature: 160 °C	0.1, 0.5or 1 mL/min
	Gradient measurement: (A) trichlorobenzene (B) cyclohexanone [146,147]	Nucleosil 500 silica column or Perfectsil 300 (250 mm × 4.6 mm) particle size: 5 or 10 μm oven temperature: 140 °C	0.1 mL/min or 1 mL/min
	Gradient measurement: (A) ethylene glycol monobutyl ether (B) trichlorobenzene [148–151]	Nucleosil 500 (250 mm × 4.6 mm) or Hypercarb (100 mm × 4.6 mm) particle size: 5 μm oven temperature: 140 – 160 °C	1 mL/min
	Gradient measurement: (A) decaline (B) cyclohexanone [152,153]	Polygosil 1000 (250 mm × 8 mm) or Perfectsil 300 (250 mm × 4.6 mm) particle size: 10 or 5 μm oven temperature: 140 °C	1 mL/min
Esters	Gradient measurement: (A) THF/H_2O (B) THF [157]	Alltech Kromasil C18 (150 mm × 4.6 mm) oven temperature: 30 °C	
	Gradient measurement: (A) $CHCl_3$ (B) EtOH [165]	Kromasil silica gel C18 column (150 mm × 4.6 mm) Particle size: 5 μm Oven temperature: 25 °C	1 mL/min
Glycans	Gradient measurement: (A) 0.1% formic acid and 10% H_2O in CH_3CN (B) 0.1% formic acid and 40% H_2O in CH_3CN [161]	HILIC-PLOT column (2.5 m × 10 μm) particle size: 5 μm	20 nL/min
Non-digestabile carbohydrates	Mobile phase: H_2O [162]	Two TSK-GEL G2500PW_{XL} (300 mm × 7.8 mm) coupled with TSK Guard Column PW_{XL} (400 mm × 6 mm); temperature: 80 °C	0.5 mL/min
Sulphonated lignins	Gradient measurement: (A) 0.05 g/L ion-pair agent in H_2O (B) 0.05 g/L ion-pair agent in CH_3CN/H_2O (90/10) [163]	Phenomenex Spherisorb ODS column (250 mm × 1.0 mm) particle size: 5 μm oven temperature: 35 °C	50 μL/min
Cellulose derivatives	Gradient measurement: (A) CH_3CN (B) H_2O [164]	Phenomex Luna C18 (250 mm × 4.6 mm) particle size: 5 μm oven temperature: 30 °C	1 mL/min

by Gorbunov et al. [182]. Due to the different combination possibilities the authors could clearly show that the combination of LAC or LCCC with SEC resulted in the best separation behavior and that the simulated plots are in good agreement with the real ones. The following parts will provide an overview about the LC × SEC measurements for synthetic polymers as well as biopolymers since 2005.

In order to establish the critical conditions for PMA, Gao et al. employed a normal phase column and a mobile phase combination consisting of methyl ethyl ketone (MEK) as "good" solvent mixed with cyclohexane as non-polar "weak" solvent for MA [75]. After determination of the best measurement parameters, linear PMA-*b*-PS block copolymers containing the same PS block length as well as star-shaped block copolymers with a three arm PS were used for the evaluation of the critical point since they elute at the same retention time according to the theoretical expectation. Furthermore, 2D measurements were performed with these linear and star-shaped block copolymers to prove the initiator efficiency of the corresponding macroinitiator, which seemed to be very high due to non-existing macrointiator signals in the 2D contour plot. Raust et al. prepared a continuative study about acrylate homo- and copolymers performing gradient HPLC and 2D-LC [126]. As first step, gradient HPLC measurements were implemented for different (meth)acrylate homopolymers, *i.e. iso*butyl, *iso*bornyl or their methacrylates, and by the use of a stepwise gradient the HPLC settings were optimized; in this case HPLC represented the

Table 6
Liquid chromatography conditions applying isocratic measurements.

Polymer	HPLC solvent mixture	Column	Flow rate
PEO	12.63/87.37 (v/v) (acetone/H_2O) [168]	Several RP columns	0.5 mL/min
PEO	24.61/75.39 (v/v) (MeOH/H_2O) [166]	Several RP columns	0.5 mL/min
PI	53/47 (v/v) (CH_2Cl_2/CH_3CN) [85]	Kromasil C18 RP column (150 mm × 4.6 mm) particle size: 5 μm oven temperature: 23 °C	0.5 mL/min
PMMA	50/50 or 70/30 (v/v) (THF/toluene) [176,177]	Silpearl (250 mm × 4 mm and 300 mm × 7 mm) particle size: 7 or 10 μm oven temperature: 30 °C	0.5 or 1 mL/min
PS	50/50 or 70/30 (v/v) (THF/toluene) [175–177]	Silpearl (250 mm × 4 mm and 300 mm × 7 mm) particle size: 7 or 10 μm oven temperature: 30 °C	0.5 or 1 mL/min
PVAc	70/30 (v/v) (THF/toluene) [177]	Kromasil (250 mm × 4 mm and 300 mm × 7.5 mm) particle size: 10 μm oven temperature: 30 °C	1 mL/min
PET, PBT, PLA	Screening of different solvent combinations [179]	Kromasil (250 mm × 4 mm and 300 mm × 7.5 mm) particle size: 10 μm oven temperature: 30 °C	1 mL/min
PS-*b*-PMMA	Different compositions of THF/toluene [180,181] screening of different solvents [178]	Silpearl (250 mm × 4 mm) and Kromasil (300 mm × 7.5 mm) particle size: 10 μm oven temperature: 30 °C	1 mL/min

best possible separation method. In order to identify exactly the different components in the 2D contour plots, a composition of different homopolymers were first analyzed by 2D-LC and, afterwards, the knowledge of the previous run was adopted on the contour plots of the random copolymer samples. Reingruber et al. investigated an alternative sample-introduction technique for GPEC × SEC measurements for PMMA samples to avoid breakthrough of the sample in the first dimension [32]. Due to the solvent mixing step directly after the autosampler it was possible to reach a clear separation of the PMMA homopolymers of different molar masses. Inglis and Barner-Kowollik utilized a two-dimensional setup as characterization tool for the efficiency and purity of their poly(methyl methacrylate-*b*-*iso*bornyl acrylate) (PMMA-*b*-P*i*BoA) copolymers synthesized *via* a conjugation reaction [103]. Applying the critical conditions for *i*BoA, the 2D contour plots of the block copolymers as well as the corresponding parent homopolymers could be prepared and the quantitative analysis of the copolymer samples indicated very low amounts of the P*i*BoA and no detectable amounts of the PMMA homopolymer, which are in good agreement with the results of the SEC deconvolution (see Fig. 4).

The successful formation of 3-arm star copolymers consisting of PS and P*i*BoA *via* "click reation" was proven by Wong et al. using 2D-LC as characterization technique under critical conditions of P*i*BoA [104]. The 3D illustration showed the clear separation of the PS homopolymer and the clicked star copolymer. Sequenced two-dimensional liquid chromatography was performed by Berek and Šiškova. combining LC LCD with SEC for the analysis of PS-*b*-PMMA block copolymers [183]. Combining HPLC in the gradient mode as first separation technique and SEC as second one, van der Horst and Schoenmakers could clearly demonstrate the separation of PS-co-PMMA copolymers from their corresponding homopolymers in a fully automated orthogonal separation procedure [7].

Beside the analysis of poly(acrylate) homo- and copolymers, also grafted and comb-like copolymers, such as poly(ethylene oxide-*g*-methacrylic acid) (PEO-*g*-PMAA) or poly(ethylene oxide-*g*-vinyl alcohol) (PEO-g-PVAlc), were investigated by 2D-LC. Pasch et al. described the separation and identification of PEO-*g*-PMAA copolymers from non-grafted PEO by 2D-LC and LCCC/Fourier transform infrared spectroscopy (FT-IR) using an aqueous mobile phase in both dimensions for the first time [184]. Additionally, two grafted copolymer species could be identified containing different amounts of MAA. Due to the use of an uncommon eluent, modifications of the stationary phases as well as the use of electrolytes or ion-pairing reagents had to be done to reach a good separation. This optimization procedure was published in a continuative study using anionic (meth)acrylate copolymers as well as grafted copolymers containing PEO and PMAA or PVAlc, which can be utilized as tablet coating materials for the controlled release of active compounds [94]. The comparison of the 2D contour plot of a graft copolymer with the 2D contour plot of the PEO precursor indicates clearly that the graft copolymer contains no free PEO species (see Fig. 5).

Instead of applying the CAP for PEO, Knecht et al. investigated the critical conditions for PVAc to separate PEO-*g*-PVAc copolymers [120]. In addition, optimization of the 2D setup accelerated the analysis time reaching an appropriate experimental time for routine applications. Similar to the LCCC investigations for different polymer classes, also 2D-LC experiments were not only performed for copolymers consisting of acrylates or oxides but also for the class of styrenes, which will be the focus in the next section. Due to the change of the operating temperature the separation of non-, mono-, and difunctionalized PS polymers synthesized by nitroxide-mediated polymerization (NMP) was performed by Petit et al. using dimethylformamide (DMF) as pure eluent and, subsequently, two-dimensional chromatography measurements enabled the determination of the living chain fraction, which could be confirmed by additional electron spin resonance (ESR) measurements [52]. By combining a specific solvent mixture, consisting of dichloromethane (CH_2Cl_2) and chloroform ($CHCl_3$), and a higher oven temperature for the LCCC partition, branched PS samples could be separated according to the branching number by Im et al. using LCCC coupled with SEC [81]. In order to achieve a better resolution of the 2D contour plot, reversed phase temperature gradient interaction chromatography (RP-TGIC) coupled with LCCC was employed additionally and allowed an improved molar mass separation. Solvent and temperature gradient chromatography coupled with SEC was applied for linear and star-shaped PS by Gerber et al. [129,130]. Due to the optimized temperature gradient the separation of the linear chains from the star polymer was possible by on-line and off-line 2D chromatography. Combining the LCCC technique off-line with SEC, Takano et al. could clearly separate cyclic telechlic poly(styrene) from linear PS chains formed during the cyclization reaction [110]. In comparison to the previous investigations for star-shaped PS samples, Edam et al. used molecular-topology fractionation (MTF) coupled with SEC to gain more information about the degree of branching [185]. Applying the CAP for PS. Schmid et al. could separate remaining PS homopolymers from a PS-*b*-PCL block copolymer by using 2D-LC [107]. Additionally, this characterization technique helped the authors to confirm the block copolymer structure and the efficiency of the synthetic process. This new polymerization procedure for sulfur-free block copolymers is based on the method of switching well-defined thiocarbonyl thio capped (reversible addition fragmentation chain transfer (RAFT)) polymers into hydroxyl terminated species. Im et al. reported a clear separation of different kinds of mikto-arm PS-*b*-PI copolymers from the corresponding PS and PI homopolymers using LCCC in combination with

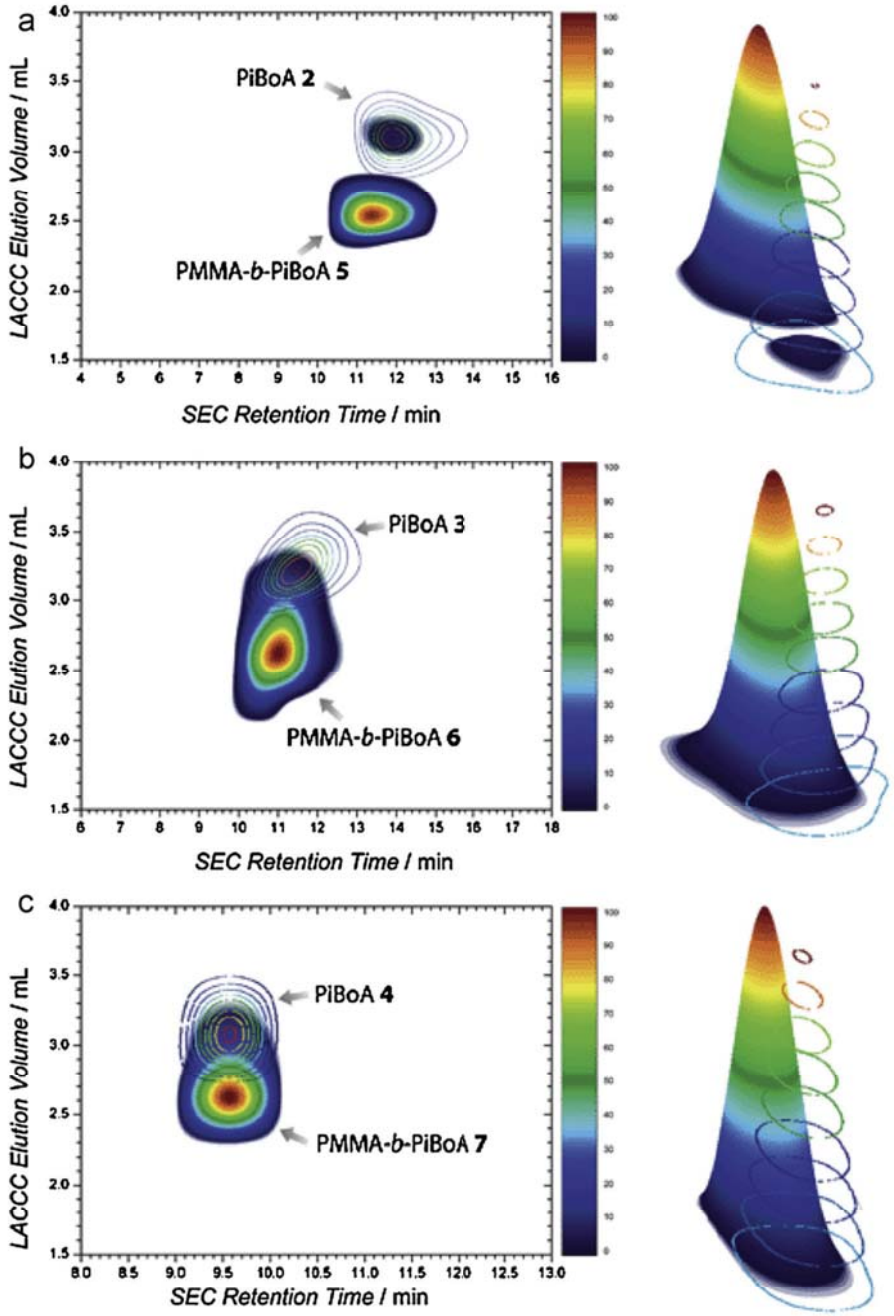

Fig. 4. 2D LACCC–SEC chromatograms and corresponding three dimensional renderings of (a) PMMA-*b*-PiBoA 5, (b) PMMA-*b*-PiBoA 6 and (c) PMMA-*b*-PiBoA 7; all overlayed with their respective PiBoA precursors.
Reproduced from Ref. [103].

HT-SEC at 110 °C [35]. Applying coupled chromatographic techniques facilitated the detection of side products formed during the copolymerization of PS-*b*-PEO block copolymers by NMP, which was studied in detail by Beaudoin et al. [186]. The 2D contour plot delivered four different species using the critical conditions for PS, which could be identified as the desired block copolymer and minor by-products, such as PEO-*b*-PS-*b*-PEO, residual macroalkoxyamine and PS homopolymer. Additional, LCCC-NMR investigations confirmed these findings. Also Min et al. used the coupled characterization techniques LCCC × SEC as analysis method to approve their new developed block copolymer preparation method, which is performed in miniemulsion by atom transfer radical polymerization (ATRP) using activators generated by electron transfer (AGET) [108]. Due to the more robust initiation step in the miniemulsion process in comparison to the normal ATRP initation step, it was possible to synthesize linear and star PMA-*b*-PS copolymers without the occurrence of the PS homopolymer and only a small amount of coupling products, which was proven by 2D-LC (see Fig. 6).

In comparison to the previous LC × SEC studies applying ELS as detection unit, Kok et al. employed an infrared (IR) flow cell coupled to the SEC as detector for a better separation of polymers according to their functional groups [187]. The authors implemented this detection possibility for a selective investigation of methacrylate in the 2D contour plot of the used styrene-methacrylate copolymers. Additionally, UV detection was used to verify the obtained results.

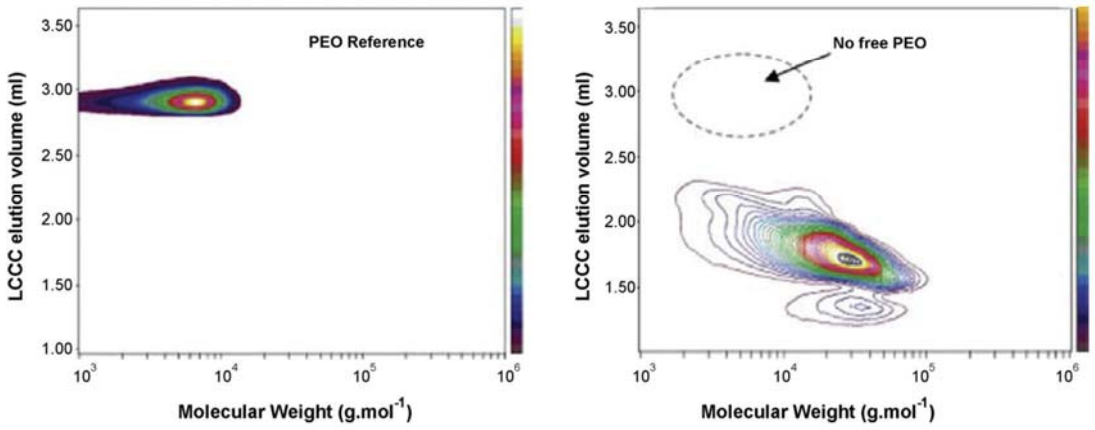

Fig. 5. 2D-LC separation of PEO-*g*-PVA copolymer 2. 1st dimension: LCCC. 2nd dimension: SEC. calibration: PEO. Reproduced from Ref. [94].

Besides the 2D-LC separation procedure at lower temperatures, several groups established the usage of high-temperature two-dimensional liquid chromatography (HT 2D-LC) for crystalline (co)polymers instead of commonly used temperature rising elution fractionation coupled with SEC (TREF × SEC). First investigations for the separation of poly(olefin)s, like a series of poly(ethylene-*co*-octene) (PE-*co*-POc) random copolymers with varying ethylene octene content, were performed by Roy et al. using HT 2D-LC at operation temperatures from 140 up to 175 °C [188]. Due to the application of 140 °C as operation temperature for the complete 2D setup, Ginzburg et al. could separate a broad variety of poly(olefin) copolymer blends consisting of PE, PVAc and ethylene-vinyl acetate (EVAc) independent on the crystallinity of the samples in contrast to the TREF × SEC results [146]. Analyzing different elastomers, namely ethylene-propylene-diene (EPDM) terpolymers, with HT 2D-LC revealed a clear separation of the copolymers in relation to the ethylene or the diene content [137]. Ginzburg et al. extended their research using HT 2D-LC and TREF × SEC to a series of olefin copolymer blends containing PE, poly(propylene) (PP) with different microstructures, ethylene-propylene (EP), EPDM rubber and ethylene/1-hexene copolymers at an operation temperature of 160 °C [138]. Lee et al. applied this straightforward technique to the determination of the chemical composition and molar mass heterogeneities of poly(olefin) copolymers using infrared and light scattering detectors [145].

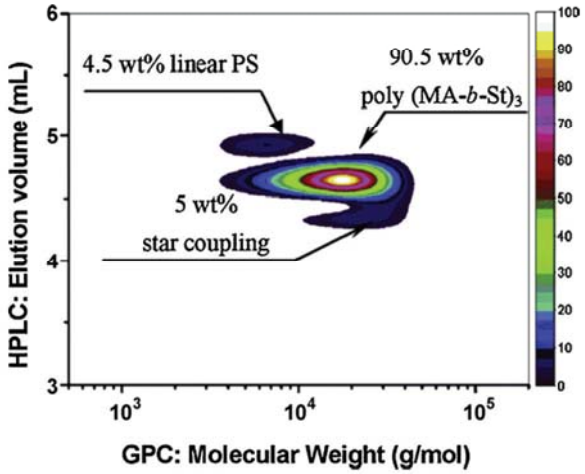

Fig. 6. 2D chromatogram of a star block copolymer poly(MA-*b*-St)$_3$ synthesized using 3-arm trifunctionalized PMA macroinitiator by SR&NI ATRP in miniemulsion. The first dimension is HPLC under critical condition for PS, and the second dimension is SEC with PS standard as calibration. Polymerization conditions: $[St]_0:[(PMA\text{-}Br)_3]_0:[CuBr_2/BPMODA]_0:[AIBN]_0 = 300:1:0.6:0.375$; 80 °C. Miniemulsion conditions: [Brij 98] = 0.58 wt% with respect to water [2.3 wt% with respect to the organic phase]; [hexadecane] = 3.6 wt% with respect to monomer. Modified from Ref. [108].

An overview about the fast and comprehensive application of two-dimensional liquid chromatography for biopolymers was provided by Stoll et al., whereby the coupling of LC with SEC or the other way around is only shortly mentioned [189]. Applying GPEC as first separation method and aqueous SEC as second dimension, Greiderer et al. could clearly demonstrate the different separation behavior regarding the molar mass, the molecular composition and the polydispersity of different HPMC polymers [160]. In addition, the impact of the column temperature on the retention time was investigated to check the gelation properties of the different compounds. Beside the investigations for HPMC polymers, GPEC combined with SEC was also applied to separate PVAc grafted onto different cellulose derivatives from the corresponding PVAc homopolymers as well as biodegradable poly(ester)s consisting of 3-hydroxyvalerate and 3-hydroxybutyrate [164,165]. One example in the field of LC at critical conditions coupled to SEC was reported by Mass et al. [80]. The authors described the separation and quantification of PS-*b*-PI block copolymers in terms of composition, total molar mass, molar mass of the individual blocks and the existance of possible by-products utilizing critical conditions for PS as well as PI. Fig. 7 displayed the complete separation of PS-*b*-PI and its corresponding homopolymers in a 2D contour plot.

3.2. Liquid chromatography coupled with liquid chromatography (LC–LC)

Besides the commonly used LC(CC) × SEC system, also two different kinds of liquid chromatography are often combined, such as RPLC × NPLC, LCCC combined with LAC or LEAC.

Most of the studies dealing with combined LC systems are applied for poly(ethylene oxide) copolymers. First investigations for the separation of PEO-*b*-PPO block copolymers were performed by Jandera et al. using a RPLC × NPLC setup [133,134]. Applying gradient conditions in the first dimension and isocratic conditions in the second dimension it was possible to separate the different PEO-PPO (co)oligomers first regarding the PO content and secondly regarding the EO content. Abrar et al. investigated LCCC at the CAP of EO combined with hydrophilic interaction chromatography for the separation of amphiphilic polymers, such as different kinds of poly(sorbate)s and fatty esters of PEO [101,190]. Further investigations for the separation of fatty alcohol ethoxylates were performed by Raust et al. using gradient LC in combination with LCCC (critical conditions for EO) [191]. Due to the different isolation methods

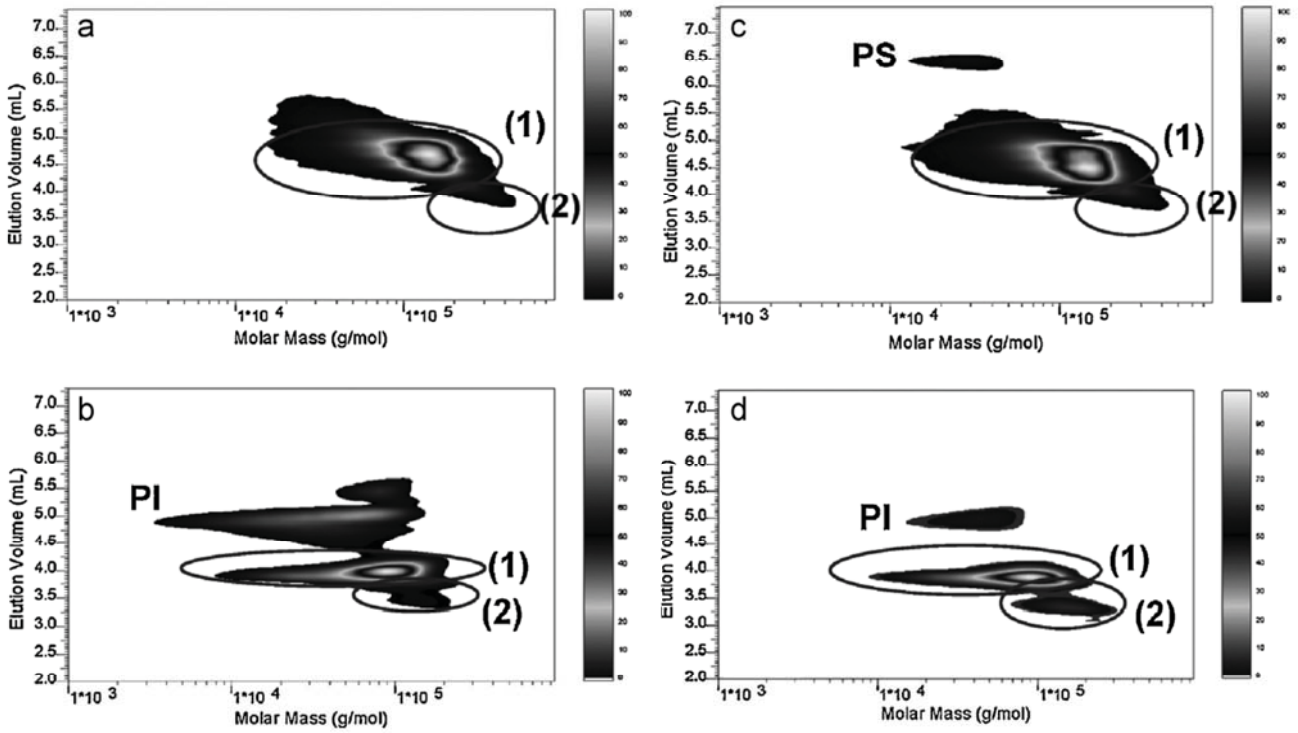

Fig. 7. 2D contour diagrams of samples *Coup PS-b-PI (18–60)* (a and b) and *Seq* PS-*b*-PI *(20–60)* (c and d), 1st dimension: LCCC (PS) (a and c) or LCCC (PI) (b and d), 2nd dimension: SEC.
Reproduced from Ref. [80].

it was possible to differentiate between the varying oligomers in length and end group functionality. A combination of LCCC and LEAC as separation possibility was applied by Ahmed et al. to distinguish between diverse PEO-*b*-PCL block copolymer fractions with varying PCL content [46].

First LC × LC measurements for PS samples were performed by Toups et al. to separate different oligomers first according to the molar mass and in the second dimension according to the structure of the oligomers [192]. In the field of PS containing block copolymers, Im et al. employed NPLC × RPLC measurements in combination with a trap column between the two dimensions to achieve a better separation of PS-*b*-PI block copolymers according to their chemical composition [85]. Applying the critical conditions for PS or PBMA in the first and the CAP of PMMA in the second dimension, van Hulst et al. used a LCCC × LCCC setup to separate different block and random copolymers, such as PS-*b*-PMMA (see Fig. 8), PBMA-*b*-PMMA and PBMA-*r*-PMMA, from the corresponding homopolymers [79].

LC × LC measurements were also performed for biopolymers. One example is the separation of sulphonated lignins adding an ion-pair agent tetrapentylammonium bromide to the mobile phase [163].

3.3. LCCC hyphenated with different mass spectrometry techniques

To overcome the lack of appropriate standards for the calibration of the SEC system, mass spectrometry techniques, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS), can be utilized as second characterization technique. Applying MALDI-TOF MS as coupling possibility, the molar mass dispersity of the (co)polymer has to be narrow (the polydispersity index (PDI) should be below 1.2–1.3), which represents the major drawback of this technique. Unfortunately, also the operation procedure can only be performed semi on-line or off-line by spotting or spraying the different LCCC fractions onto the MALDI target. But nevertheless, MALDI-TOF MS can provide important additional information about the polymer structure, such as the composition of the copolymer or the nature of the end groups, in comparison to SEC. The latest publications about LC hyphenated with MS were summarized shortly by Peacock et al. [193] and Weidner et al. in their mass spectrometry reviews about synthetic polymers [194,195].

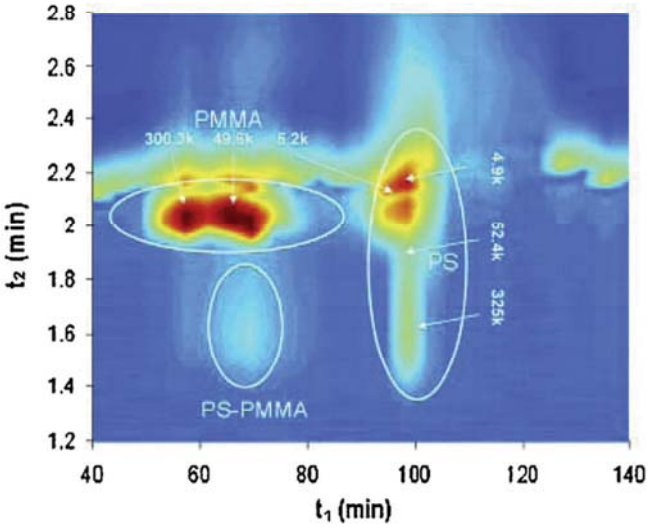

Fig. 8. CC × CC contour plot of homopolymers of PS (4.9, 52.4 and 325 kDa) and PMMA (5.2, 49.6 and 300.3 kDa) and styrene-methylmethacrylate block copolymer (blocks of 62 kg/mol S and 62 kDa MMA).
Reproduced form Ref. [79].

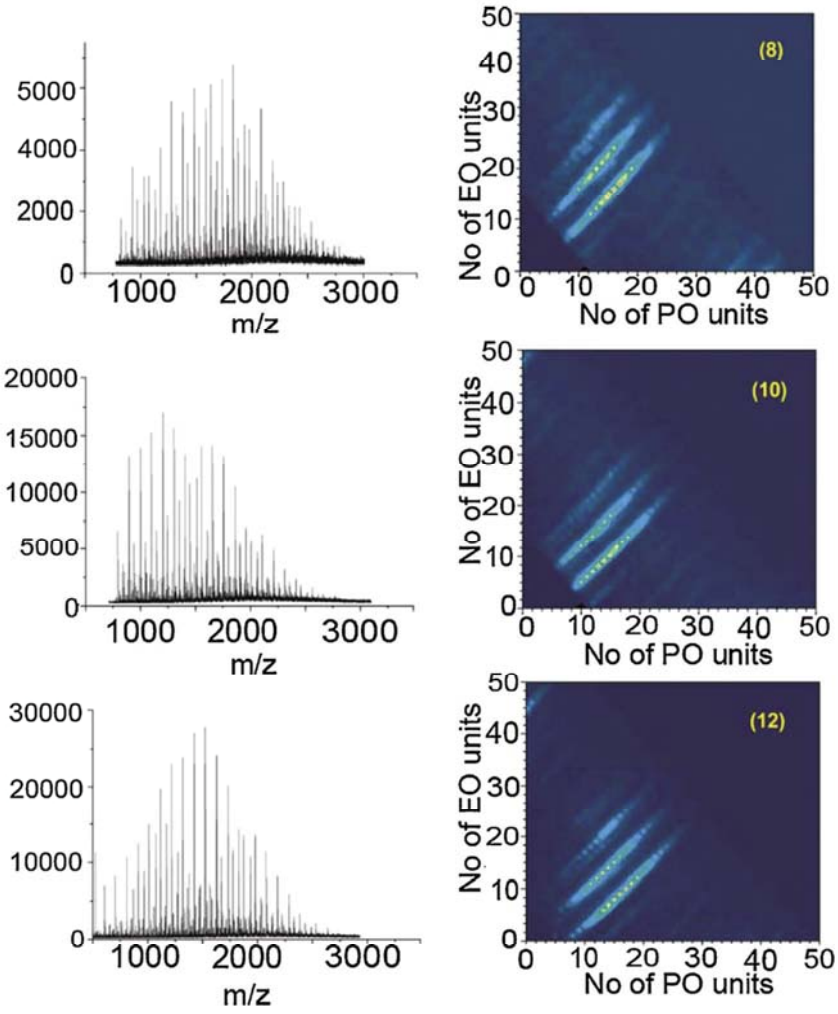

Fig. 9. MALDI-TOF mass spectra (left) and corresponding 2D composition plot (right) of fractions 8, 10 and 12 recorded after separation in isocratic chromatography. Reproduced from Ref. [67].

Weidner et al. used a combination of LCCC and MALDI-TOF MS for the separation and investigation of a polyester copolymer [123]. Collecting the LCCC fractions manually and spotting them afterwards onto the matrix precoated MALDI target or performing the 2D separation (semi) on-line by spraying the LCCC run directly onto the target, it was possible to determine the exact copolymer composition knowing the end group structure. For a faster interpretation of the copolymer data after LC–MS separation, the authors developed later on a new software tool "MassChrom2D" to determine the composition of PEO-*b*-PPO block copolymers [67]. MALDI-TOF MS spectra with the corresponding 2D composition plot generated by the new software tool are displayed in Fig. 9. Due to the existence of isobaric copolymer structures with the same m/z value in the MS spectrum, the additional performance of tandem mass spectrometry (MS/MS) on different spots in the 2D plot demonstrated clearly the existence of only one isobaric copolymer structure.

Malik et al. used the coupling procedure of LCCC with MALDI-TOF MS in an off-line process to better understand the formation of PEO-*b*-PPO block copolymers applying anionic ring opening polymerization of PO by using PEO as initiator [66]. Besides the formation of the desired diblock copolymer, also PPO homopolymers with allylic or hydroxy end groups were found, which can be formed either by chain transfer reaction during the polymerization or due to traces of water present in the initial polymerization mixture. To overcome the inhomogeneous "dried-droplet" spotting technique, which is commonly used for the MALDI-TOF MS sample preparation, Falkenhagen and Weidner utilized a spraying technique for sample preparation [99]. This new solvent-free sample preparation method allowed also the direct coupling to chromatographic systems, such as SEC or LCCC. In this study, a clear separation and identification of PEO polymers with different end groups was possible and resulted in MALDI-TOF MS spectra with higher reproducibility and sensitivity due to a better homogeneity of the samples. Further investigations in the field of solvent-free sample preparation were reported by Trimpin et al. for different functionalized PEO homopolymers [100]. Using a custom-made sample holder, simultaneous and homogenous on-target multisample solvent-free sample preparation was applied to reach high sensitivity detection of the fractionated polymers. Beside MALDI-TOF MS, Falkenhagen and Weidner also used ESI-TOF MS as on-line coupling technique to liquid chromatography [93]. In order to shorten the characterization time, first an ultra high performance liquid chromatography (UPLC) system as first dimension was applied for the analysis of ethoxylated fatty acid alcohols and PEO-*b*-PPO block copolymers and, secondly, the previous developed "MassChrom2D" software allowed an easy and quick interpretation of the data.

Using LCCC as separation tool and MALDI-TOF MS for the verification of the polymer structure, Ahmed et al. applied these techniques to investigate the influence of the applied catalysts on the polymerization reaction of PEO-*b*-PCL block copolymers [114]. Tin octoate seems to be the best choice as initiator, because no

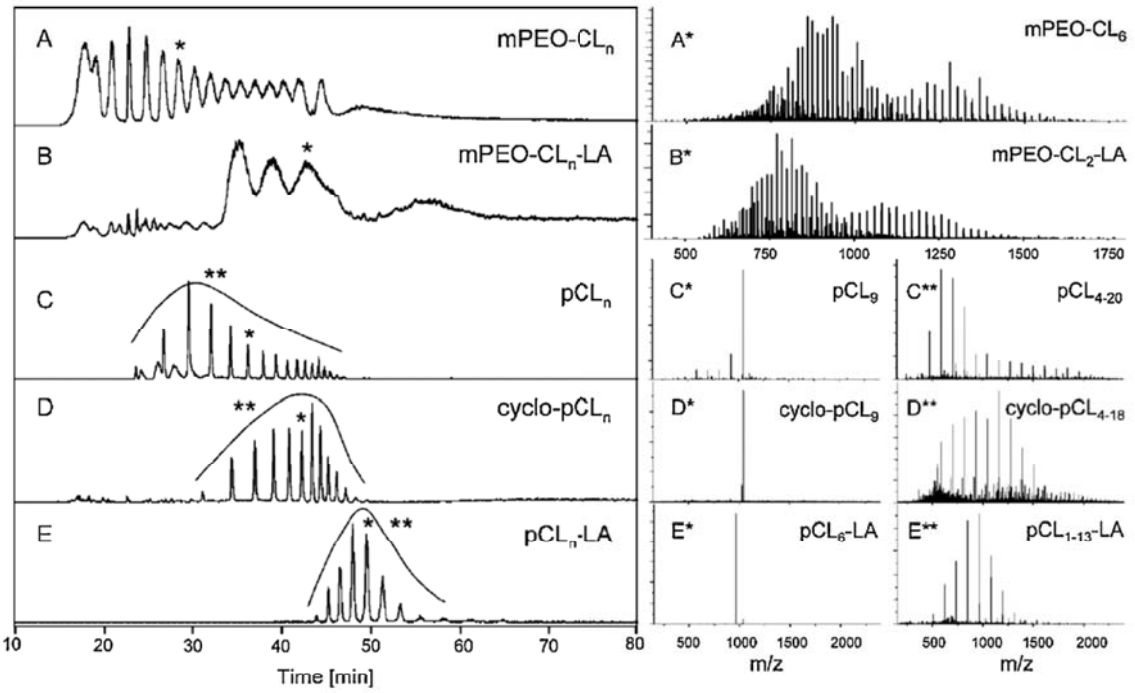

Fig. 10. LC/MS chromatograms showing the separation of the homologous polymer series contained in the block copolymer samples: (A) total ion chromatogram (TIC) of unfunctionalized polymer, recorded with ESI(+)-MS; (B) TIC of functionalized polymer, recorded with ESI(+)-MS; (C) multiple ion chromatogram (MIC) of 17 selected mass traces of unfunctionalized polymer, recorded with APCI(−)-MS; (D) MIC of 14 selected mass traces of unfunctionalized polymer, recorded with APCI(+)-MS; and (E) MIC of 13 selected mass traces of functionalized polymer, recorded with APCI(−)-MS. Examples of the corresponding mass spectra are given for the marked peaks, as well as for the complete homologous series shown in (C–E).
Reproduced from Ref. [132].

PCL-A homopolymer or cyclic PCL products appeared also after a longer heating time. On-line coupling of LCCC with ESI-TOF MS was performed for PEO-*b*-PS copolymers by Girod et al. after optimization of the critical conditions for PEO using a solvent mixture of methanol and water containing an electrospray cationizing agent, *e.g.* LiCl [96]. In this case, (co)oligomers could be separated due to their PS block length and characterized in detail by ESI-TOF MS up to seven PS repeating units. Supplementary tandem MS investigations verified the predicted copolymer structure based on the occurring fragmentation series. In a continuative study, the influence of the salt concentration in the mobile phase on the fragmentation pathway behavior was implemented for the same PEO-*b*-PS (co)oligomers [97]. Due to the occurrence of $[M+H+Li]^{2+}$ ions additionally to doubly lithiated ions, detailed structural information about the SG1 end group could be gained from the collision-induced dissociation (CID) spectrum using a lower salt content. Additionally, Girod et al. studied the salt effect in the mobile phase on the CAP of PEO coupled on-line to an ESI MS instrument [98]. Due to the nature of the different salts as well as the size of the cations, interactions with column material, *e.g.* non-polar as well as polar stationary phases, were strongly influenced during the LCCC run.

A combination of HPLC in the gradient mode and ESI MS/MS was used by Pulkkinen et al. to characterize the enzymatic degradation of oxazoline-linked poly(ε-caprolactone) by a new direct and simple procedure [131]. The tandem mass spectrometry measurements provided new important information on the enzymatic degradation process by pancreatic enzymes and the structure of the polymer. The initiation process of enzymatic ring-opening polymerization for PCL was studied by de Geus et al. using LCCC coupled with MALDI-TOF MS [115]. *Via* characterization in the off-line mode the authors could demonstrate that water dominates the initiation process whereby cyclic structures as well as linear ones with different end groups are formed. Information about the polymer backbone composition and the block length dispersity for PEO-*b*-PCL functionalized with linoleic acid was reached applying gradient liquid chromatography coupled with mass spectrometry using atmospheric pressure chemical ionization (APCI) and ESI [132]. An overview about the separation of homologous polymer series contained in the block copolymer is presented in Fig. 10.

Beside the frequently studied block copolymers containing PEO, Fandrich et al. applied hyphenated LCCC techniques to characterize amphilic block copolymers consisting of *N*-vinylpyrrolidone (VP) as well as VAc and to prove the accurateness of the polymerization mechanism *via* the RAFT process [118]. Applying critical conditions of PS enabled Byrd et al. to examine the covalent cationization method. The accurateness of the polymerization process was further proven by MALDI-TOF MS investigations [196]. Applying HPLC in the gradient mode combined with ESI MS and MS/MS enabled to obtain a complete overview about the possible initiation and termination reactions during the synthesis of PBA homopolymers [127]. Combining HPLC in the gradient mode with MALDI-TOF MS as second characterization step for PAMAM dendrimers represents an important tool for the determination of the structural defects during the synthesis [155]. Beside the already mentioned MS techniques, Kaal et al. applied RPLC measurements in the gradient mode combined with pyrolysis-gas chromatography-mass spectrometry (LC–Py-GC MS) for the characterization of different water-soluble copolymers. Based on the results of the Py-GC MS obtained from the different LC fractions, it was possible to determine the monomer composition of two different PEO-*b*-PPO and PS-*r*-PMMA copolymers [197].

Because of its high sensitivity, specificity and selectivity, HPLC coupled to (tandem) mass spectrometry is often applied as routine method for biopolymers. Thereby, the fragmentation technique delivers more specific information about the chemical nature of the polymer and its end groups. Ridlova et al. developed a LC/ESI–MS

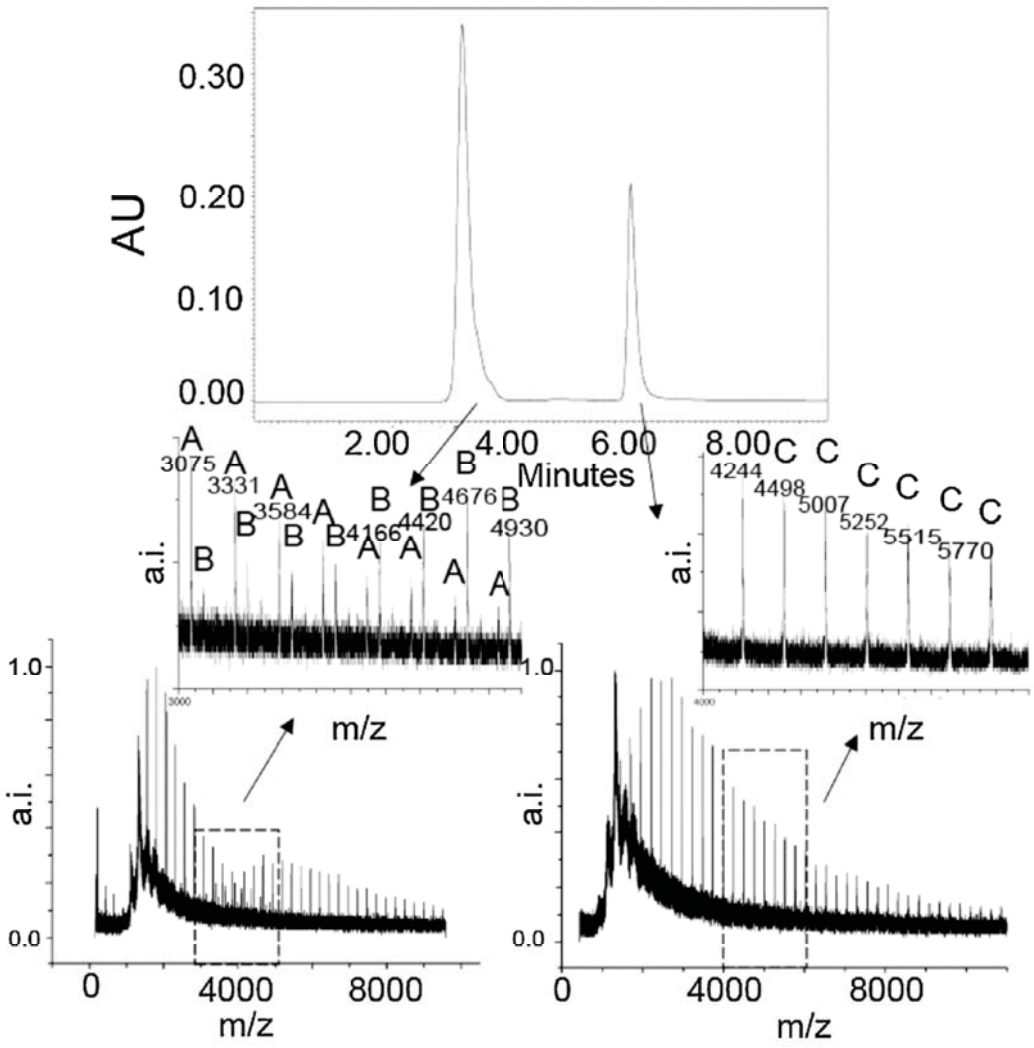

Fig. 11. Identification of the peaks observed with LCCC using semi on-line coupling of MALDI-TOF MS. Reproduced from Ref. [113].

platform (OliQuIT) for profiling oligosaccharides derived from glycosyl hydrolase digestion of polysaccharides [158]. In addition, also very minor components can be identified by this platform to reach important information about the glycosyl hydrolase specifity and the arabinoxylan structural diversity in different plant series. Applying NP-HPLC coupled off-line with MALDI-TOF MS and tandem mass spectrometry, identification of arabinoxylan structures as well as structural information about the dispersity of arabinofuranose side chains and extensive linkage information could be reported by Maslen et al. in their publication about arabinoxylan oligosaccharides [159]. Due to its low sensitivity for the analysis of glycans using nano-LC hyphenated with ESI MS/MS, Luo et al. developed special HILIC-PLOT columns for the separation of glycans at the ultratrace level with a ten times higher ionization efficiency [161]. Reproducible preparation and application could be reached with a HILIC-PLOT column at a very low flow rate of 20 nL/min. Gradient HPLC combined with ESI-TOF MS and MS/MS in the positive as well as negative mode provided an optimal overview about the degradation process of poly(ester)s, possible cyclic side products as well as the copolymeric sequence of biodegradable poly(esters) consisiting of butylene adipate and butylene terephthalate monomer units [157]. The characterization of copolyesters using liquid chromatography at critical conditions combined with different mass spectrometry techniques was performed by Weidner et al. [123,198]. The application of tandem mass spectrometry confirmed the occurrence of ring and linear oligomeric structures with different end groups consisting of hexanediol adipic acid and neopentylglycol adipic acid, which could be separated by LCCC.

3.4. Combination of LC–SEC and LC–MS

The combination of LC–SEC and LC–MS measurements was frequently used to confirm the structure of copolymers and to investigate the polymerization process. Malik et al. applied LCCC in the first dimension and afterwards SEC, LAC or MALDI-TOF MS in the second dimension in the off-line mode for higher polyoxyalkylene block copolymers, such as PEO-*b*-PBO or PEO-*b*-PHO [66,69]. The final characterization by MALDI-TOF MS and the spectrum interpretation of the separately collected fractions during the first dimension verified the existence of by-products, such as unsaturated and diol homopolymers of PBO or PHO, beside the corresponding block copolymer. Adler et al. introduced aqueous mobile phases for the separation of water-soluble copolymers by two-dimensional chromatography techniques and LCCC hyphenated with MALDI-TOF MS [95]. In addition to the investigation of the CAP for PEO the starting material MPEO as well as the macromonomers PEO-MM was analyzed by LCCC and MALDI-TOF

MS. Moreover, also comb-like copolymers of EO and MAA were used for further LCCC/FT-IR and 2D-LC studies to separate the copolymer from its polymerization by-products and residual homopolymer.

Functionalized acrylate polymers were analyzed by Jiang et al. applying comprehensive two-dimensional chromatography (LC × SEC) [12]. Due to the slightly change of the mobile phase composition (CH_3CN/methylene chloride) near the CAP of PMMA it was possible to identify and separate non-, mono- and difunctionalized PMMA polymers from each other. Additionally, qualitative results of the RAFT synthesized PMMA polymers were confirmed by on-line LC–ESI MS measurements and, thus, the existence of different end groups could be proven.

Beside the critical conditions for commonly studied polymers, such as PEO, PMMA or PS, Coulier et al. evaluated the CAP for poly(bisphenol A carbonate) (PC) to separate virgin PC from aged PC samples [113]. Due to the combination of LCCC with SEC it was possible to differentiate between these two polymer types. In addition, the change of the SEC detection unit from an UV based system to an IR flow cell allowed to obtain qualitative information about the time-dependent degradation process of PC polymers from the 2D contour plot more easily and secondary MALDI-TOF MS measurements of the LCCC fractions confirmed the end group change from *t*-butyl (B) to OH (C) during the degradation process (see Fig. 11) [111,112].

Baumgaertel et al. applied a combination of LCCC and SEC for the characterization of a poly(2-oxazoline) block copolymer [119]. Performing additional LC–MS measurements at the CAP of PEtOx, homopolymer side products could be clearly separated from the corresponding block copolymer and, secondary, tandem MS investigations delivered further information about the structural background. Julka et al. used a combination of two-dimensional liquid chromatography (SEC × LCCC) and ESI-TOF MS for the quantitative characterization of solid epoxy resin components. Beside the composition of the polymer, also the conditions for different polymerization techniques could be established and used as information for verifying molecular architecture models for the production of solid epoxy resins [117]. Gutzler et al. utilized LCCC × SEC measurements of PEO-*g*-PVA copolymers to confirm the absence of PEO homopolymer [199]. Additional MALDI-TOF MS measurements and morphology studies verified these results and, therefore, the synthesized grafted copolymers were suitable for applications as instant release tablet coating.

4. Conclusion and outlook

A larger number of investigations were performed in the last years to introduce LCCC as powerful analysis tool for the characterization of complex synthetic (co)polymers as well as biopolymers beside the commonly applied LAC, LEAC or LC LCD methods. This meaningful technique was mainly adopted for a complete separation of polymer samples, like poly(alkylene oxide)s, poly(acrylate)s or other synthetic polymers, with different functionalities independent of their molar mass. Furthermore, different models as well as complete prediction tools were developed to predict the critical point of adsorption without the enormous laboratory effort. In addition, the combination of at least two autonomous systems, such as LCCC × SEC, LC × LC, LCCC × LC or hyphenation of LCCC with different mass spectrometry techniques, delivers further information about the polymeric sample due to their independent separation possibility, such as chemical composition or functionality on the one side and molar mass or architecture on the other side. Beside deeper insights of the polymer architecture, also additional information about the polymerization process itself or the initiator efficiency can be gained comparing two-dimensional contour plots of either the traditional polymerization route or the improved process. Unfortunately, applying all necessary steps to reach the mentioned degree of information this characterization procedure is very time, laboratory effort and cost consuming. But nevertheless, using comprehensive polymer analysis techniques will be one important enrichment for academic as well as industrial applications in the future due to its enormous capability, in particular for copolymers for pharmaceutical and medical applications.

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