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Uniform Macromolecules: Performance of Common Analytic Instruments in Detecting Impurities

*Philipp Bohn, Maximiliane Frölich, Daniel Hahn, Rebekka V. Schneider, and Michael A. R. Meier**

Inspired by the highly defined structure of biomacromolecules, e.g. deoxyribonuleic acid (DNA) and proteins, the preparation and characterization of sequence-defined and uniform macromolecules gained interest in polymer chemistry. With the development of various synthetic approaches, the challenge of analyzing and confirming the uniform structures emerged. Here, the investigation on the performance of common analytical instruments for the characterization of uniform macromolecules regarding impurities of ±1 in degree of oligomerization is presented. Thus, different mixtures containing oligomers differing in one repeating unit of oligo(ethylene glycol)s (OEG)s, oligo(phenylene ethynylene)s (OPE)s, and Passerini oligomers, respectively, are synthesized, and analyzed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR), size exclusion chromatography (SEC), and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). These results demonstrate the indispensability of a careful characterization using more than one technique for determining uniformity. In particular, the obtained SEC results show that it is a powerful tool for purity determination of low molecular weight oligomers.

1. Introduction

Since Hermann Staudinger published the first article on polymerization 100 years ago, a milestone has been set for the world of synthetic polymers.[1,2] Since then, a plethora of polymerization techniques were investigated, resulting in polymeric materials that we can no longer lack in our everyday life. However, traditional polymer chemistry, including conventional step- and chain-growth polymerization, has always been the science of dispersity and distribution. To achieve higher molecular control, reversible deactivation radical polymerization (RDRP)[3–5] like atom transfer radical polymerization (ATRP),[6,7] nitroxide-mediated polymerization (NMP),[8–10] and reversible addition-fragmentation chain-transfer polymerization (RAFT),[11–13] as well as living anionic polymerization approaches[14] were introduced. The ability to adjust the dispersity opened the opportunity to tune polymer properties and thus triggered the synthesis of tailor-made materials.[15–18] With the development of these polymerization techniques, the advancement of characterization methods became necessary. Besides nucelar magnetic resonance (NMR) spectroscopy[19] and mass spectrometry,[20] size exclusion chromatography (SEC is nowadays probably the most important analysis method for polymers.[21] However, in order to determine exact structure-property relationships, uniform polymers are inevitable. Pioneering work in this direction was performed by Robert B. Merrifield with the development of the Solid-Phase-Peptide-Synthesis (SPPS) in 1963,[22] for which he received Nobel Prize in 1984.[23] Since then, research in the field of preparing uniform or sequence-defined synthetic macromolecules has gained much interest in recent years. Several methods, including solid-[24–27] and liquid phase,[28–31] or solid supported synthesis,[32] single unit monomer insertion (SUMI),[33] or template assisted synthesis were investigated.[34] These approaches are based on different iterative concepts: unidirectional or bidirectional growth, exponential growth (IEG), or chain tripling.[35] Here, we applied the unidirectional growth for the synthesis of the oligo(phenylene ethynylene)s (OPEs) and Passerini oligomers, as was reported by our group in the recent years.[31,36–39] For the preparation of oligo(ethylene glycol)s (OEGs) via an IEG strategy, we performed a comparative study in 2020 and applied the gained knowledge recently for the preparation of uniform block cooligomers.[30,35] The accessibility to synthesize tailor-made structures allows first insights into their structure-property relationships and the creation of new

P. Bohn, M. Frölich, D. Hahn, R. V. Schneider, M. A. R. Meier
Laboratory of Applied Chemistry
Institute of Organic Chemistry (IOC)
Karlsruhe Institute of Technology (KIT)
Kaiserstraße 12, 76131 Karlsruhe, Germany
E-mail: m.a.r.meier@kit.edu

M. A. R. Meier
Laboratory of Applied Chemistry
Institute of Biological and Chemical Systems – Functional Material Systems (IBCS-FMS)
Karlsruhe Institute of Technology (KIT)
Herrmann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

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compounds for potential application in material and life science.[40–45] However, the synthesis of uniform macromolecules also comes with the obligation of confirming their purity, which is not always provided as we highlighted in a review in 2017.[40] A thorough characterization, which should include at least a liquid chromatographic method (either high pressure liquid chromatography (HPLC) or SEC equipped with appropriate columns), high resolution mass spectrometry (HR-MS), and NMR as well as reports of synthesis procedures are often incomplete.[40] Thus, the term "uniformity" (or "monodispersity") is often used inflationary and incorrectly. Especially for applications in the pharmaceutical sector, but also for the establishment of structure-property relationships, a uniform structure and a distinct structure-property relationship is crucial.

In 2009, Davis et al. described the challenge of synthesizing uniform poly(ethylene glycol) (PEG) with $M_n > 20$ kDa to achieve an enhanced in vivo serum half-life from commercially available PEG building blocks due to insufficient purity. They showed a statistic modeling of three different PEG samples: a benchmark PEG with a purity of 98.8%, a commercial amino-dPEG$_{24}$ *tert*-butyl ester with a purity of 96%, and a synthesized amino-PEG$_{16}$ methyl ether with a purity of 99.8%. Furthermore, the latter two compounds were compared in electrospray ionization mass spectrometry (ESI-MS) to another commercially available PEG 750, which demonstrated the high purity and thus a low dispersity of the self-prepared sample.[46] Bruce et al. analyzed a PEG sample that was prepared according to the procedure of Davis et al.,[46] as well as Tanaka et al.[47] with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and compared it to a PEG synthesized via their established method.[48] The results highlighted the efficacy of the approach to obtain uniform PEGs. In addition, a comparison of ESI-MS and MALDI-MS of a PEG$_{12}$ was performed and led to the conclusion that MALDI-MS is probably the only suitable method capable to distinguish PEGs differing in only one repeating unit. Moreover, literature-described sample decomposition under MALDI-MS conditions as well as side reactions with the used matrices were not observed, thus purity quantification of the PEG was possible.[48–51] In the same context they describe the limitations of SEC for the determination of uniformity on the basis of an equimolar mixture of PEG$_{20}$ and PEG$_{21}$, which showed only one signal in the chromatogram (c = 10.0 mg mL^{-1}, 2 × mixed bed-E, 30 cm, 3 mm). However, the study of Kinbara et al. on a chromatography-free synthesis of uniform PEGs demonstrated the high resolution of reverse phase (RP)-HPLC, where clear signals up to a PEG$_{31}$-Ts were observed.[52,53] Since PEG is applied in various different areas, where a uniform structure is beneficial, the determination of the purity was investigated thoroughly. However, this is not the case for non-natural sequence-defined macromolecules, which became an emerging research field in the last decade,

Here, we demonstrate the synthesis of three different uniform macromolecules (n-mers) containing up to 15 wt.% of impurity ((n-1)-mers) and their characterization via ^{1}H NMR, SEC, and HR-ESI-MS. SEC with THF as eluent was preferred over HPLC due to its versatility in terms of sample solubility (OPEs for instance are poorly soluble in MeCN) and the simple operation in isocratic mode, thus avoiding the often necessary customized methods for different types of compounds in HPLC analysis. The focus of this study is on the determination of the uniformity of the individual compounds and the advantages and limitations of commonly available and applied characterization methods in polymer chemistry.

2. Synthesis of Oligomers

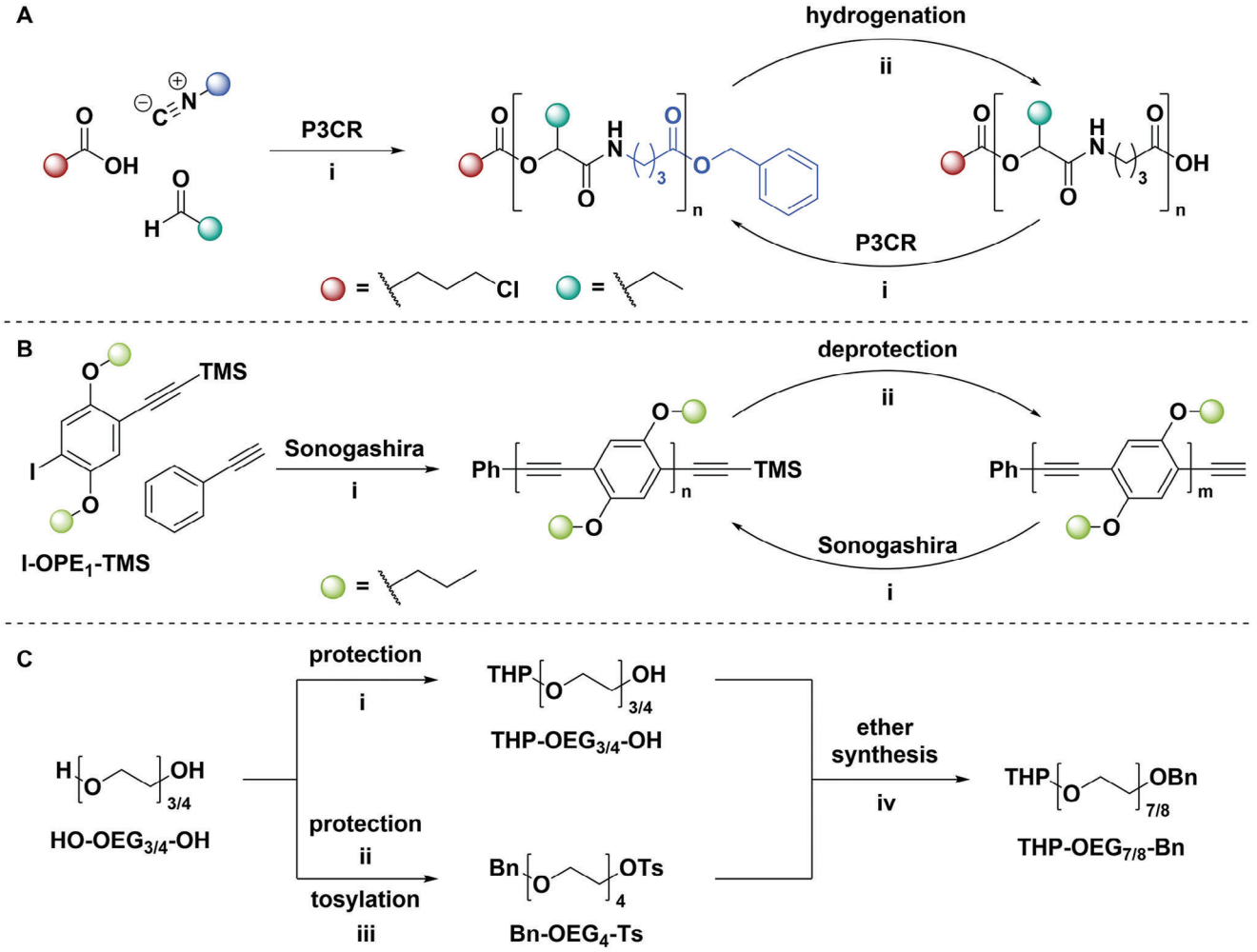
The synthesis of the uniform OPEs and Passerini oligomers were performed according to an iterative two-step reaction cycle, while the OEGs were prepared via chain coupling (**Scheme 1**). The preparation of sequence-defined macromolecules according to an iterative chain elongation cycle consisting of a Passerini three-component reaction (P3CR) and a subsequent reductive hydrogenolysis was established in our group in 2014.[54] Using 4-chlorobutyric acid, propionaldehyde, and benzyl isocyanobutyrate (obtained in an overall yield of 49%, see supporting information) as starting materials, and the two latter compounds for the chain elongation, a tetramer **ClPr-P3CR$_4$-Bn** and pentamer **ClPr-P3CR$_5$-Bn** were prepared in an overall yield of 30% and 22%, respectively (Scheme 1A).

An OPE trimer **PhAc-OPE$_3$-TMS** and tetramer **PhAc-OPE$_4$-TMS** were obtained via an iteration of a Sonogashira cross-coupling reaction and a subsequent TMS deprotection (Scheme 1B).[55] Phenyl acetylene was thus reacted with building block **I-OPE$_1$-TMS**, which was obtained from hydroquinone in a three-step procedure (29% yield, see supporting information),[56–58] yielding the monomer **PhAc-OPE$_1$-TMS**. Afterwards, the silyl ether was cleaved under basic conditions and the obtained alkyne was used in a Sonogashira reaction with **I-OPE$_1$-TMS**. By repetition of this reaction cycle, the trimer **PhAc-OPE$_3$-TMS** was obtained in eight reaction steps and 16% overall yield, and the corresponding tetramer **PhAc-OPE$_4$-TMS** in 10 steps and a yield of 10%.

The third to be analyzed compound pair are orthogonally protected hepta- and octa(ethylene glycol), which were synthesized via an ether coupling of the monobenzyl tetra(ethylene glycol)tosylate **Bn-OEG$_4$-Ts** and the corresponding THP protected building block **THP-OEG$_{3/4}$-OH** (Scheme 1C).[59] Therefore, the respective ethylene glycol diols **HO-OEG$_{3/4}$-OH** were monoprotected, and the benzyl protected tetramer **Bn-OEG$_4$-OH** was transformed into the activated tosylate **Bn-OEG$_4$-Ts** by treatment with *p*-toluenesulfonyl chloride.[35]

All products were purified via column chromatography, and the reaction scales, yield and product identification (NMR, infrared (IR) spectroscopy, ESI-MS) are provided in the supplementary information and the respective publications.[35,55,60] For the OEGs, where elimination during the Williamson ether synthesis is the most common side reaction and further formed side products were detected by SEC-ESI-MS in a previous work,[35] the products were purified two times via column chromatography (see Tables S8, and S9, Figures S15, and S16, Supporting Information). In total, 28 individual fractions were taken, where the ones showing the narrowest distribution were used for this purity study.

To investigate the advantages and limitations of typically applied analytical methods for oligomer characterization, the respectively higher DP oligomer with a chain length of n repeating units (i.e., **PhAc-OPE$_4$-TMS**, **ClPr-P3CR$_5$-Bn**, and **Bn-OEG$_8$-THP)** was contaminated with the (n-1) oligomer (**PhAc-OPE$_3$-TMS**, **ClPr-P3CR$_4$-Bn**, and **Bn-OEG$_7$-THP**, respectively)



Scheme 1. Approaches for the synthesis of the individual uniform oligomers. A) Preparation of Passerini macromolecules by reacting 4-chlorobutyric acid, propionaldehyde and benzyl isocyanobutyrate in a P3CR (i: DCM, rt, 24 h) and subsequent iterative two-step elongation cycle consisting of a reductive hydrogenation (ii: Pd/C, H_2, EA, rt, 24 h) and a Passerini reaction with propionaldehyde, benzyl isocyanobutyrate; B) Preparation of uniform OPEs via a Sonogashira reaction of phenyl acetylene and building block I-OPE_1-TMS (i: $PdCl_2(PPh_3)_2$, CuI, Et_3N, THF, rt, 24 h) and subsequent chain elongation cycle consisting of a deprotection (ii: K_2CO_3, DCM/MeOH, rt, 12 h) and a Sonogashira reaction with I-OPE1-TMS; C) Synthesis procedure toward uniform OEGs via a divergent protection steps i) 3,4-Dihydro-2H-pyran (DHP), *p*-TsOH, DCM, rt, 24 h; (ii) BnBr, $NaOH_{aq}$, 100 °C, 20 h) and activation of the alcohol (iii) *p*-TsCl, NaOH, THF/H_2O, rt, 15 h) and subsequent convergent ether coupling (iv: KO*t*Bu, THF, rt, 20 h); TMS = trimethylsilyl, Ph = phenyl, Bn = benzyl, Ts = tosyl.

in amounts between 1–15 wt.%. All analytical measurements (^{1}H NMR, SEC, and HR-ESI-MS) were performed starting from the same mixtures, and the procedure for the preparation of the individual samples is provided in the supplementary information. Observed and expected trends are reported for each analytic technique, where a slope of 1.0 and an ordinate intersection of 0.0 would represent a perfect agreement between theoretically expected and actually determined impurity content.

2.1. Analysis via ^{1}H NMR

^{1}H NMR is one of the most common and powerful tools in an organic chemistry laboratory for the identification of a compound's chemical structure and its purity. In this study, ^{1}H NMR was used for structural confirmation and proton assignment of the different oligomers. However, due to possible precursor impurities still present from the iterative synthesis protocols, which only differ in the amount of the backbone protons, thus showing a similar signal pattern in NMR, determination of the compound's purity by NMR is challenging. A comparison of the ^{1}H NMR spectra of the different mixtures of n and n-1 oligomers are shown in **Figure** 1A–C. The decrease of the backbone integrals with increasing amount of impurity of the three different investigated oligomer types is very difficult to distinct without careful integration (Figure 1A–C). For all oligomer series, all possible changing NMR integral ratios were evaluated for their suitability to determine impurities of n-1 oligomers. For the OPE oligomers, this corresponds to the protons of signals 3, 4, 5 and 6, for the P3CR oligomers to the signals 2, 3, 6, 8, 10 and 11, and for the ethylene glycol oligomers to the signal 5 (see supporting information for assignment). Graphical representations of the detected amount

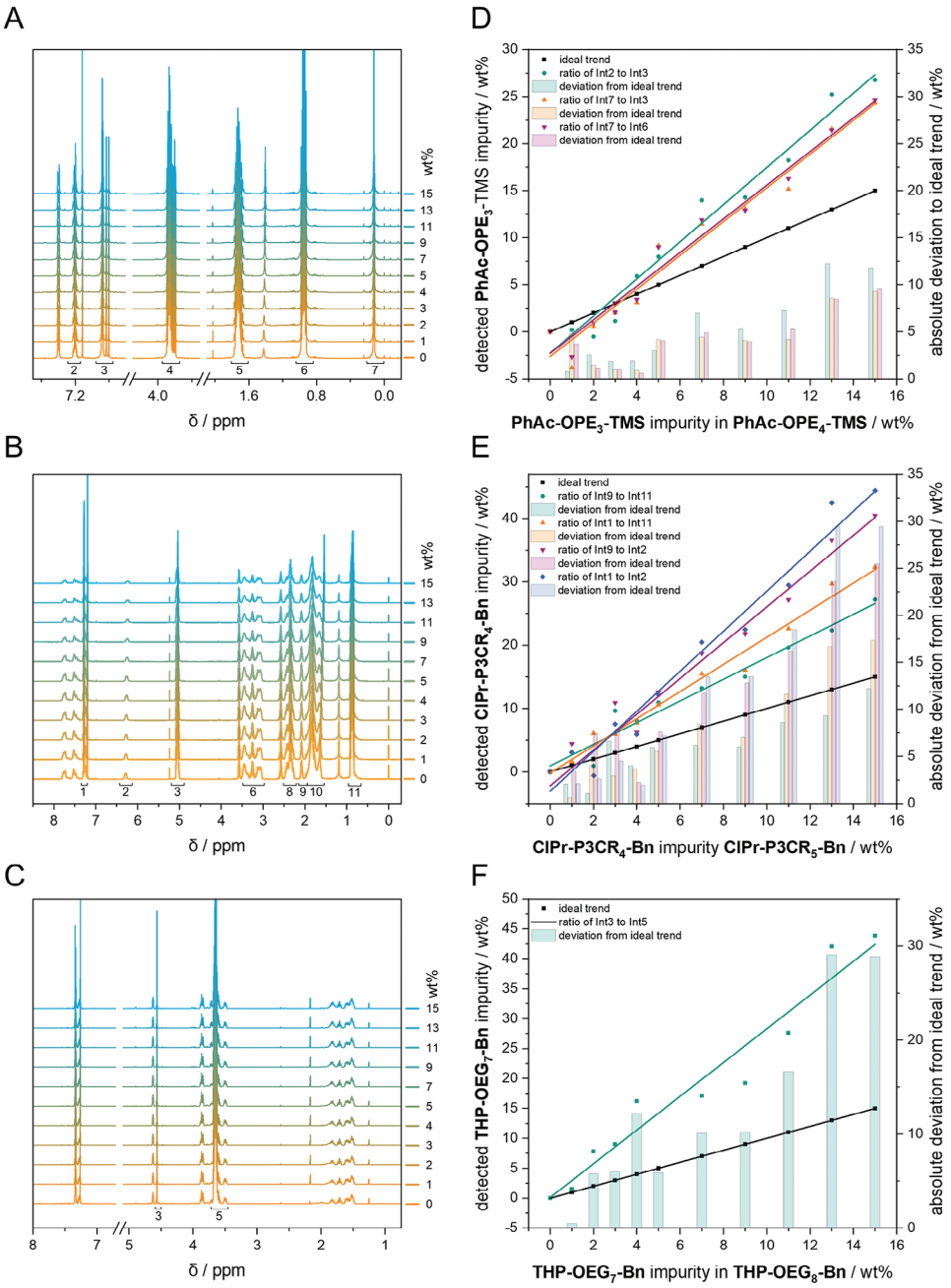


Figure 1. Impurity detection via ^{1}H NMR spectroscopy. A–C) Comparison of ^{1}H NMR spectra of the oligomer mixtures containing up to 15 wt.% of the (n-1)-mer. E,F) detected amount in wt% (formular (1) supporting information) of impurity obtained from different integral ratios and absolute deviation from the ideal trend in wt% (color coded).

of impurity for the three oligomer compound pairs are illustrated in Figure 1D–F. The values were obtained from the raw ^{1}H NMR data applying formula (1) (see supporting information) and the ideal trends are represented in black. Figure 1D shows the trends for the OPEs obtained by comparing the aliphatic end group integral of the TMS-protons (Int7) with either the aliphatic CH_3 backbone integral (Int6, violet trend) or with the aromatic CH backbone integral (Int3, orange trend), and the comparison of both aromatic signals Int2 with Int3 (green trend). All integration details are provided in the supplementary information. The trends show slopes of 1.79 ± 0.10 (violet trend) to 1.97 ± 0.11 and absolute deviations ranging from 0.81 to 12.2 wt.% (green trend, 1 wt.% impurity). From the available data, ratio Int7 to Int3 is the most reliable to determine the oligomeric impurity. For the P3CR oligomers, we conducted the comparison of either aliphatic CH_2 (Int9) or aromatic CH (Int1) end group signal to the aliphatic CH_3 (Int11) and the amide NH (Int2) backbone signals. The corresponding trends (Figure 1E) related to the comparison

with the aliphatic backbone methyl group deviate from the ideal trend with an increased slope of 1.71 ± 0.12 (green trend) and 2.14 ± 0.09 (orange trend). However, a deviation of 12 wt.% was detected for the green trend at 15 wt.% impurity. On the other hand, the trends obtained from the comparison with the backbone amide (blue and violet trends) deviate more strongly from the ideal due to peak broadening and probably deuterium proton exchange in $CDCl_3$ resulting in a decreased integral. It is thus important to carefully select suitable signals for an impurity evaluation. For the OEGs (Figure 1F), the only varying integral is the one of the CH_2 ethylene backbone signal (Int5). Since the aromatic peaks are overlapping with the solvent signal and the integral of the THP CH_2 and CH protons are surprisingly fluctuating between values of 6.69 and 7.66 or 0.82 and 1.02, respectively, when referenced to the CH_2 benzyl signal (Int 3), the latter one was compared to Int5 (green trend). The obtained values highly deviated from the ideal trend showing an absolute deviation of 29 wt.% of determined impurity for the samples containing 13 and 15 wt.% of THP-OEG_7-Bn and of −4.1% for the sample with 5 wt.% impurity.

In conclusion, ^{1}H NMR is a powerful and inevitable technique to identify the molecules' structure. However, to quantify small amounts of oligomeric impurities, which only differ in the integrals of the backbone protons, is quite challenging and requires a careful selection of suitable peaks to integrate and evaluate. For the OEGs, no such peak could be identified, thus ^{1}H NMR seems unsuitable to determine the purity of OEGs. Furthermore, a pure sample is always necessary as reference, which was especially challenging for the uniform OEGs.

2.2. Analysis via SEC

After the NMR measurements, the $CDCl_3$ was removed under reduced pressure and three samples in a concentration of 2 mg mL^{-1} in THF were prepared for SEC analysis and measured directly one after the other. The average chromatograms of the sample mixtures are shown in **Figure** 2A–C and the curves are superimposed at their maximum (**PhAc-OPE_4-TMS** x_{max} = 18.22 min, **ClPr-P3CR_5-Bn** x_{max} = 18.49 min, **Bn-OEG_8-THP** x_{max} = 19.83 min). The difference of molecular mass of one repeating unit is 216 Da for the OPEs, 171 Da for the P3CR oligomers, and 44 Da for the OEGs. Depending on the mass difference of the two respective compounds and thus the change of the hydrodynamic volume, either a separate peak (Figure 2A), a shoulder (Figure 2B), or a peak broadening (Figure 2C) was observed with increasing amounts of the impurity. Especially for the OEG mixtures, these measurements are close to the resolution limit of the instrument and thus the detection of the impurity percentage was the most challenging (OEG: $\Delta x_{max} = 0.21$ min, compared to OPE: $\Delta x_{max} = 0.54$ min, P3CR: $\Delta x_{max} = 0.36$ min). In a previous study, we demonstrated the identification of a 2 wt.% impurity via SEC using a peak symmetry evaluation.[20] Due to overlapping of the signals, a peak deconvolution would deliver more exact results. However, by evaluating the total integral of the different mixtures and comparing the value to the pure compounds, the increase of the amount of impurity could be calculated in a more straightforward fashion using formula (2) (see supporting information). The corresponding trends are shown in Figure 2D–F and compared with the ideal trend in black. For the OPE mixtures, a trend with a slope of 1.26 ± 0.02 and a y-offset of 0.74 ± 0.14 is obtained, i.e., slightly decreased compared to the results obtained from the ^{1}H NMR analysis. For the P3CR oligomers, the trend strongly deviates from the ideal trend with a slope of 2.07 ± 0.02, and y-offset of −0.77 ±0.08 and thus a deviation of 68% for the mixture containing 15 wt.% impurity, matching the results from the ^{1}H NMR analysis. Although the values are not accurate and a quantification is not possible, already a 2 wt.% impurity could be detected in the SEC chromatograms, also for the OPE oligomers, by simple visual inspection, whereas for the PEGs a contamination can only be suspected from 7 to 9 wt.%. However, despite the minor difference in retention time of **THP-EG_7-Bn** and **THP-EG_8-Bn**, and the large error for the ^{1}H NMR results, the observed trend fits the expected trend surprisingly well, demonstrating that the quantification via the peak integral is possible and an alternative to the previously discussed peak symmetry evaluation.[35] A trend with a slope of 0.98 ± 0.01 and a y offset of 0.26 ± 0.12 was observed, yielding reliable values for low percentage impurities with deviations of 0.10 to 0.77 wt.% in the mixtures containing up to 5 wt.% impurity, which is the most interesting range in determining the uniformity of a compound.

In conclusion, a purity quantification via SEC is possible for low molecular weight oligomers, even for compounds with a small molecular weight difference of 44 Da and the corresponding small change in hydrodynamic volume. However, as well as for NMR analysis, a chromatogram of the pure substance is necessary as a reference. Furthermore, the SEC resolution depends on different parameters, such as the pore size of the column material, concentration, temperature, and the size and difference in the hydrodynamic volume of the macromolecules. In this context, Bruce et al. reported the SEC comparison of PEG_{20} and PEG_{21}. An equimolar mixture was shown, demonstrating the indistinguishability of them, and describing mass spectrometry as the perhaps only analytical method which is capable to do so.[48]

2.3. Analysis via HR-ESI-MS

HR-ESI-MS of the different samples was measured as third analytical method. A comparison of the mass spectra of the n-mer and the mixture containing 15 wt.% of the (n-1)-mer for the three compound classes under investigation (OPEs, P3CR oligomers and OEGs) are shown in **Figure** 3A–C, respectively. The individual single charged adduct ion signals ($[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, and $[M+K]^+$) are labeled accordingly and additionally highlighted in blue for the (n-1)-mers. For the OPEs mixtures shown in Figure 3A (top), a significantly lower ionization efficiency was observed as for the P3CR oligomers and OEG, resulting in a lower signal to noise ratio. Since the ion signal is proportional to the analyte concentration, under certain circumstances, e.g., the concentration limit of the detector or ionization efficiency of the compound, ESI-MS is suitable for quantitative analysis. Typically, an internal standard, similar to the structure of the analyte, is required.[61] However, since all samples were prepared with a known concentration of the analytes, no additional internal standard was added, as the n-mer can be considered as internal standard. Furthermore, due to the structural

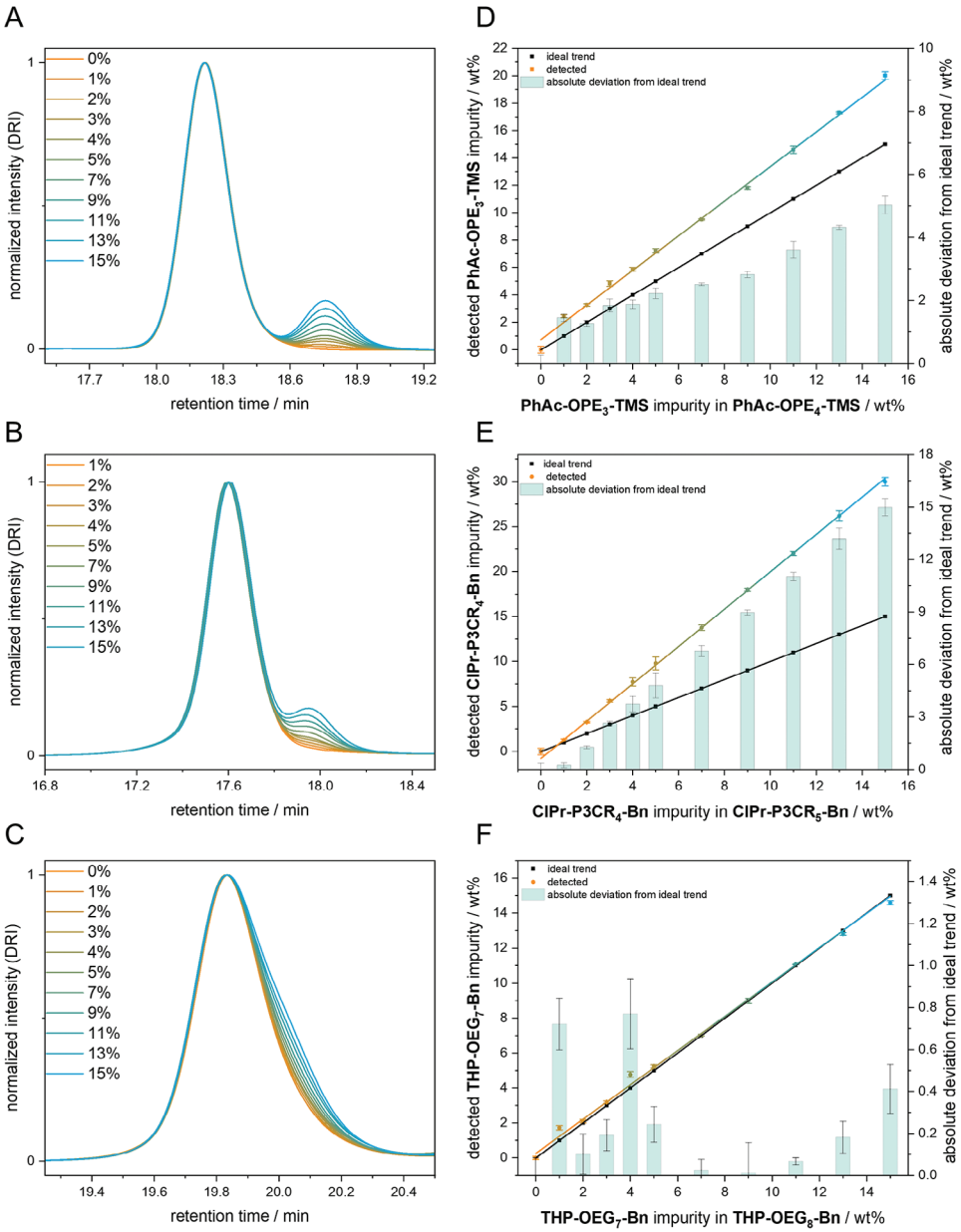


Figure 2. A–C) SEC chromatograms and integral evaluation of the different compound mixtures (1–15 wt.% impurity). The chromatograms were superimposed at their maximum (A) PhAc-OPE_4-TMS containing PhAc-OPE_3-TMS impurities, B) ClPr-$P3CR_5$-Bn containing ClPr-$P3CR_4$-Bn, C) THP-OEG_8-Bn containing THP-OEG_7-Bn impurities) and the integrals were calculated and normalized to the uniform sample D–F) color coded and compared to the ideal trend (black) and absolute deviation in wt% from the ideal trend in green.

similarity of individual oligomer pairs, the ionization efficiency, which is strongly compound dependent, was neglected. First, the spectra of the pure n-mers (**PhAc-OPE_4-TMS**, **ClPr-$P3CR_5$-Bn** or **THP-OEG_8-Bn**) were evaluated if traces of the precursor oligomers (**PhAc-OPE_3-TMS**, **ClPr-$P3CR_4$-Bn** or **THP-OEG_7-Bn**) could be observed. The amplified sections of the different spectra are provided in the supplementary information, which show the advantages of mass analysis over NMR and SEC analysis. Already in the samples considered pure, minor signals for the (n-1)-mer were observed for the P3CR of 2.57 wt.% for the $[M+H]^+$ adduct and 0.98 wt.% for the $[M+Na]^+$ adduct, as well as for the OEGs of 0.06 wt.% for the $[M+Na]^+$ signal. Thus, according to the official definition of a "uniform polymer" by the IUPAC, **ClPr-$P3CR_5$-Bn** and **THP-OEG_8-Bn** can be declared as uniform compounds only related to SEC analysis.[62] On the other hand, no precursor signal ($[M+H]^+$ adduct) for the **PhAc-OPE_4-TMS** was detected, which is why this sample can be considered as truly uniform. A detailed assignment of the individual (n-1)-oligomer adduct peaks ($[M+H]^+$ for the OPEs, and $[M+Na]^+$ for the P3CR as well as the OEGs) are shown in Figure 3D–F, respectively. An increase of the signals, normalized to the corresponding n-mer, with increasing wt% of the (n-1) oligomer contamination

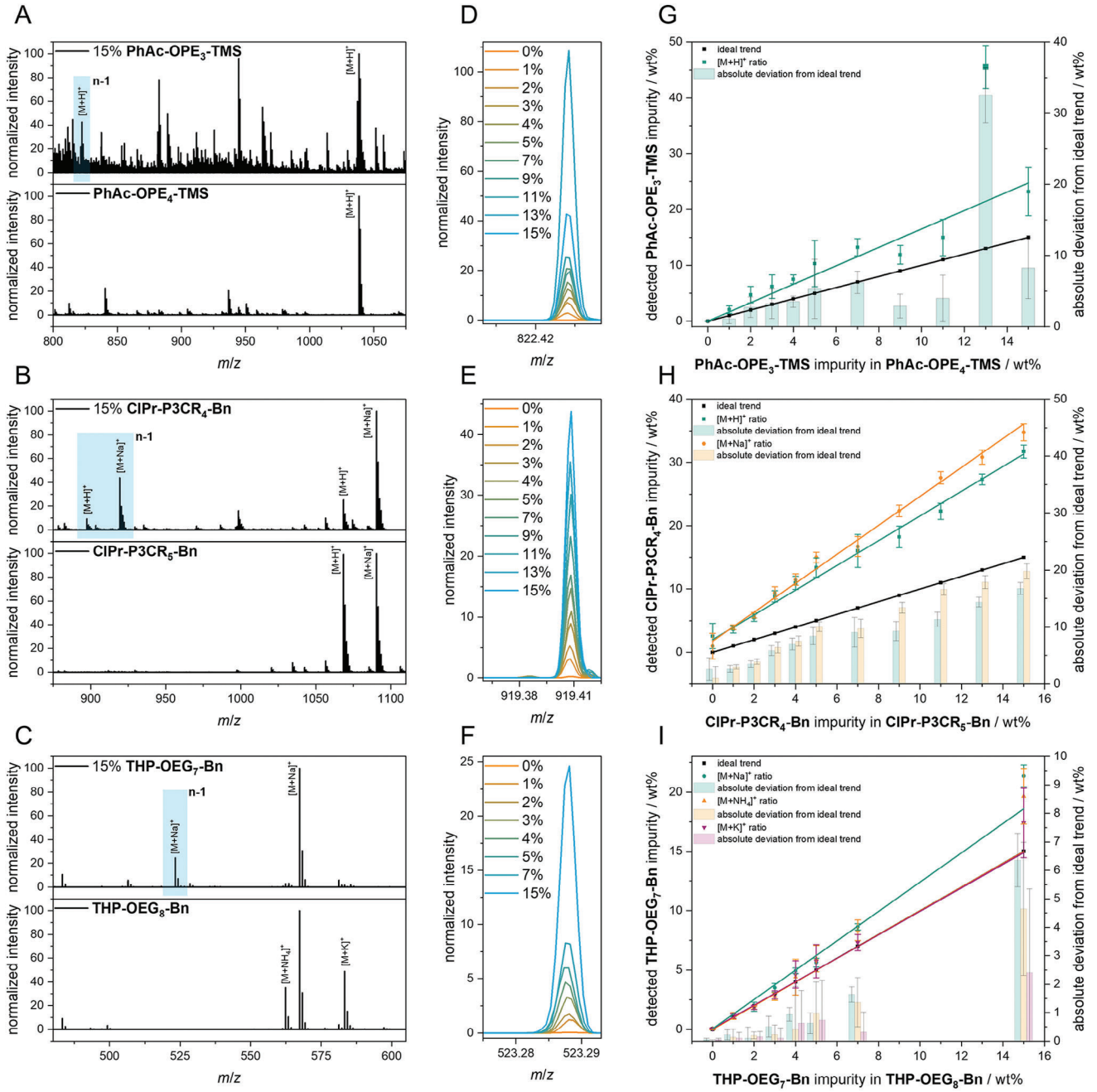


Figure 3. HR-ESI-MS analysis of the different oligomer mixtures. A–C) Comparison of mass spectra of the individual pure n-mer and the mixture containing 15 wt.% of the (n-1)-mer; D–F) Zoomed area, showing the increasing (n-1)-mer peak with increasing amount of the (n-1)-mer content. The peaks are normalized to the corresponding n-mer signal of the same ion adduct; G–I) Trends for the individual ion adduct ratios (color coded) of the different oligomer pairs compared to the ideal trend in black and the absolute deviation in wt% from the ideal trend (color coded).

was clearly observed. The corresponding trends of the individual ion adduct ratios are presented in Figure 3G–I in comparison to the ideal trend in black. The values for the different mixtures were calculated using the obtained values of the 10 scans of each mass measurement and applying formular (3) (see supporting information). Although the ionization efficiency for the OPEs was comparable low, an accurate trend was observed for the comparison of the $[M+H]^+$ adduct ratio, which was the only detected ion adduct (Figure 3G, green trend). The value of the mixture containing 13 wt.% of the (n-1) oligomer was not considered for the calculation of this trend. However, a slope of 1.65 ± 0.1, a y-offset of 0.004 ± 0.04 and absolute deviations of 1.08 wt.% for the mixture containing 1 wt.% and 8.21 wt.% for the mixture containing 15 wt.% of the oligomer impurity were observed. For the P3CR oligomers, the precursor molecule was already observed for the pure sample with amounts of 0.98 ± 2.03 ($[M+Na]^+$

ratio, Figure 3H, orange trend) to 2.57 ± 1.99 wt.% ($[M+H]^+$ ratio, green trend). The corresponding trends calculated from the two ion adduct ratios are almost identical in their slope of 1.95 ± 0.05 (green trend) and 2.29 ± 0.06 (orange trend). For the OEGs (Figure 3I), the trends obtained from the $[M+NH_4]^+$ and $[M+K]^+$ ion adduct ratio fit the ideal trend with a slopes of 1.00 ± 0.06 (orange trend) and 0.99 ± 0.04. However, in contrast to the other two oligomer pairs, the mixtures containing 9, 11, and 13 wt.% of the (n-1)-oligomer were not measured, since cleaning the instrument between each measurement was challenging, influencing the observed ratios. This might be an explanation for the increased value for the sample containing 15 wt.% impurity. On the other hand, slightly increased values for the trend of the $[M+Na]^+$ adduct ratio was obtained (slope of 1.14 ± 0.04 and y off-set of 0.05 ± 0.05). However, overall, all three trends follow almost the ideal trend and a quantitative statement about the products' uniformity is possible.

The results demonstrate the advantage over the previously described analytical methods (NMR and SEC). The individual mixtures can be analyzed independently from each other, thus offering the opportunity to determine the sample without any contaminations. Furthermore, in principle oligomers with repeating units smaller than 44 Da are distinguishable, which might be problematic for SEC analysis. On the other hand, cleaning between the individual measurements is challenging, leading to possibly wrong peak ratios.

3. Conclusion

In summary, this study highlights the challenges in analysis and determination of the uniformity of different macromolecules differing in one repeating unit. To understand limitations of analytical techniques for different oligomer types, three types of oligomers (OPEs, P3CR compounds, and OEGs) were contaminated with varying amounts of the corresponding (n-1)-oligomer. ^{1}H NMR spectroscopy, SEC and ESI-MS were used, and their individual potentials to detect impurities were analyzed, regarding structural similarity and small mass difference of the analytes. For the OPE macromolecules, ^{1}H NMR and SEC have proven to be suitable methods for accurate determination of the molecule's purity, whereas ESI-MS was challenging, due to the low ionization efficiency, but still promising results were obtained. In contrast, quantification of the (n-1) oligomer contamination was possible for the OEGs via ESI-MS and SEC analysis, although the two compounds had only a mass difference of 44 Da, while with ^{1}H NMR spectroscopy a large deviation from the ideal trend was observed. For the P3CR oligomers unfortunately none of the applied analytical methods was suitable for quantification of the purity. However, each of the measurements allowed a statement about uniformity, but the study also shows that the determination of uniformity is not trivial, and one should be careful with claiming it.

Overall, ^{1}H NMR is a powerful technique to identify the molecules' structure, however, for the quantification of the purity for molecules similar in their structure, it is the most erroneous of the investigated ones. SEC analysis delivered the most accurate results, except for the P3CR oligomers. The advantage of ESI-MS over NMR and SEC is that no reference spectrum of the pure sample is necessary. Thus, each of the mentioned tools has its strengths and weaknesses in a certain field of application, and therefore the combination of several analytical instruments is inevitable for both the characterization of a substance and the determination of its purity.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords

HR-ESI-MS, NMR, oligo (1,4-phenylene ethynylene)s, Passerini oligomers, poly(ethylene glycol)s, SEC, uniform macromolecules

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