Hyphenation of ultra-high-performance liquid chromatography and ion mobility mass spectrometry for the analysis of sequence-defined oligomers with different functionalities and tacticity

Marie-Theres Berg, Artjom Herberg, and Dirk Kuckling
Department of Chemistry, Paderborn University, Paderborn, Germany

ABSTRACT
In recent years, sequence-defined oligomers (SDOs) gained increasing interest due to their perfectly controlled molecular structure, thus providing defined properties. In order to tune the properties, different functionalities need to be incorporated into the oligomers and the chain tacticity needs to be controlled. Beside the synthesis of SDOs, suitable methods need to be found to analyze the molecular structure. In this work, oligomers exhibiting an alternating or block-wise sequence of side chain functionalities were analyzed using a hyphenation of ultra-high-performance liquid chromatography and electrospray ionization mass spectrometry enhanced by ion mobility separation (IMS). Moieties in the side chains were varied according to polarity and bulkiness. Moreover, chain tacticity was varied. Drift times in the IMS cell and the corresponding collision cross section (CCS) values were shown to be individual parameters allowing the identification of SDOs, even in the case that SDO structures only differ in sequence or tacticity of side chain functionalities. Thus, a library of CCS values was obtained as reference used for the analysis of complex mixtures of SDOs.

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Introduction
A new and rapidly expanding research area is the synthesis of bioinspired, non-natural macromolecules with precisely determined monomer sequences.[1–3] The terminology “sequence-defined” is thereby used to describe uniform macromolecules with defined length and monomer sequences without any kind of dispersity.[4]

Different approaches for the synthesis of such macromolecules are reported, while the most eclectic ones rely on iterative couplings of building blocks.[5–12] In these, the purification step can be simplified using solid support and automatization with a liquid handling robot. This allows the purification with simple washing and filtration steps.[13] One recently introduced strategy is based on thiolactone chemistry and used for the oligomers in this work. In a two-step submonomer strategy, the immobilized thiolactone is ring-opened with an amino-alcohol and subsequently the in situ released thiol moiety is implemented with an acrylate function to introduce the side chain functionality. In the second step, the new latent thiol moiety is established using a thiolactone with an isocyanate functionality.[14–17] During this synthesis one carbon-stereocenter is integrated in the backbone of the growing chain for each introduced monomer. The resulting
tacticity of the macromolecule depends on the stereochemistry within the thiolactone structure. The use of racemic thiolactone results in atactic structures, while enantiomeric pure thiolactones allow the synthesis of isotactic macromolecules.[13]

Applications of such oligomers are envisioned in different areas. For example, mimicry of biomacromolecular behavior is realized including molecular recognition and catalysis.[18-21] A different approach for the use of sequence-defined macromolecules is the application as data storage using the monomer units as bits. Procedures using binary codes as well as codes with a “monomer alphabet” were investigated.[22-26] These molecules provide a denser information storage since the individual monomer units vary in size between 2 and 10 Å.[4]

All applications of such oligomers are in need of suitable characterization methods. To investigate the sequence of macromolecules standard methods for homo or simple copolymers [typically a combination of size exclusion chromatography (SEC) and nuclear magnetic resonance spectroscopy (NMR)] are often insufficient. A widely used method is tandem mass spectrometry (MS/MS). The macromolecules are purposefully fragmented with a subsequent read out of the sequence by the mass differences between the fragments. This method is established for a broad range of polymers.[27-30] Lutz et al. combined this method with the ion mobility separation mass spectrometry (IMS-MS) to use the drift time of the produced fragments to provide the rules for the decryption of poly (alkoxyamine phosphodiester).[31] Within IMS-MS experiments ions are separated regarding their charge state, their size, and shape, which is represented by their rotational average collision cross section (CCS).[32] The IMS-MS is an established tool to analyze, separate, and identify not only structural isomers and conformers of several polymers but also protein structures and carbohydrate isomers.[33-41]

MS/MS experiments often require a certain understanding of the sample and the occurring fragmentation process. Hence, it is often combined with a prolonged evaluation of the complex fragmentation patterns. Therefore, in our approach, the oligomer identification should take place with the hyphenation of a rapid size-exclusion chromatography system and IMS-MS. This hyphenation enables a quick and easy separation as well as identification of oligomers up to sequence isomers. In a previous work, we were able to introduce the combination of an ultra-high performance liquid chromatography (UHPLC) system and the IMS-MS to successfully separate between two hexamers with different sequences of the two used monomer units.[42] Within the UHPLC, the samples were separated regarding their hydrodynamic volume. Advantages of UHPLC compared to standard SEC systems are the higher stability against high pressures, the flexibility against changing solvents and improved resolutions due to particle sizes below 2 μm.[43] Due to the used column material these systems are resistant to high back pressures which also allows a change of solvent with a stable performance.[44]

A hyphenation of UHPLC and mass spectrometry will establish an additional separation dimension. The oligomers will be separated according to their hydrodynamic volume prior to injection into the mass spectrometer. With respect to that, the complexity of the analyte solution infused into the mass spectrometer at a certain time frame will be reduced. As a consequence, the resulting time resolved mass spectra should be less complex, allowing a better evaluation. Moreover, regarding the analysis of sequence-defined oligomers, negative phenomena of mass spectrometry (e.g. different ionization of molecules with different functionality and molar mass) might be avoided by injection of sample fractions.

We have already shown the potential of a hyphenation of UHPLC and electrospray ionization (ESI)-IMS mass spectrometry for the analysis of one hexamer pair with a block-wise and alternating sequence of two side chain functionalities.[42] In this work, the analysis is extended to oligomers of different side chain functionalities and tacticity. ESI-IMS mass spectrometry is shown to be capable of identifying oligomers, even if they only differ in monomer sequence or tacticity. UHPLC separation afforded the fractionation of complex oligomer mixtures according to the hydrodynamic volume of the oligomers prior to infusing into the mass spectrometer. The
hyphenation of UHPLC and ESI-IMS mass spectrometry was applied to the analysis 20 oligomers and two oligomer mixtures.

**Experimental**

**Materials**

Tetrahydrofuran was purchased from VWR in HPLC quality, acetonitrile was purchased from Carl Roth with LC-MS grade (99.95%). The oligomers were synthesized according to a previously reported protecting group-free two step iterative protocol.[15]

The structures of investigated oligomers are listed in Table 1.

**Characterization**

The Waters Advanced Polymer ChromatographyTM (APC) system equipped with a refractive index (RI) detector and two Acquity APC XT 45 Å columns (APC XT 45 Å 7.5 cm and APC XT 125 Å 7.5 cm) was used to perform the size-exclusion experiments.

A Waters SYNAPT™ G2 mass spectrometer with an electrospray ionization (ESI) source and a build-in traveling wave-IMS cell was used to perform the mass spectrometric experiments and the subsequent IMS investigations.

For the experiments with the hyphenated system the samples were dissolved with a concentration of 2 mg mL⁻¹ in tetrahydrofuran/acetonitrile (v/v = 7/3) with an addition of 10 mg L⁻¹ of sodium iodide. Column oven and RI detector of the APC were operated at a temperature of 30 °C. To divide the flow of the APC (flow rate set at 0.7 ml min⁻¹) the systems were hyphenated by a T-type splitter and a PEEK-SIL capillary (Ø 50 μm, length 50 cm) leading to the ESI-source. The resulting split ratio was 1/10.5 (ESI-source/RI-detector).

Stand-alone measurements of ESI-IMS-MS were performed with a further diluted sample (900/50/50, solvent/sample-solution/salt-solution). Sodium iodide was dissolved with a concentration of 2 mg mL⁻¹ in the solvent mixture. The solution was infused with a syringe pump and a PEEK-SIL capillary by-passing the infusion system of the SYNAPT™. The flow rate was set to 20 μL min⁻¹. The ESI source was operated in positive ion mode.

The samples were ionized with optimized capillary, sampling, and extraction cone voltages which are listed in Table 2. The mixtures were prepared with the mass ratio of 1/1 regarding all mixed oligomers. The containments of all mixtures are listed in Table 3.

All IMS measurements were performed with a wave height of 40 V and with varying wave velocities. The calibration of the IMS system was performed with polyaniline in a solution of acetonitrile and water with acetic acid (v/v/v 49.5/49.5/1) and a concentration of 2 mg mL⁻¹. The calibration was performed with a wave velocity of 500 m s⁻¹.

**Results and discussion**

Sequence-defined oligomers (SDOs) of different chain lengths were analyzed using a hyphenation of UHPLC and ESI-IMS-TOF mass spectrometry. The SDOs exhibited a block-wise or alternating sequence of monomers with two distinctively different side chain functionalities (Table 1). The side chain functionalities were chosen in order to represent aromatic (benzylic), heterocyclic (furfuryl), non-polar (isooctyl), polar (carboxylic acid), and bulky (isoborneol) groups.

In the first step, each of the SDOs was individually analyzed using the hyphenation of UHPLC and ESI-IMS-TOF mass spectrometry. Thus, a library of individual drift times was obtained (Figure 1(a), Table S1). The SDOs comprising four monomers (tetramers) occurred as singly charged ions, whereas the SDOs with six and eight monomers (hexamers and octamers) were
Table 1. Structures of the 10 isomer pairs used in this work.

| Structure | Diagram |
|-----------|---------|
| BT₂       | ![Diagram](image1) |
| B₂T₂      | ![Diagram](image2) |
| BT₃       | ![Diagram](image3) |
| B₃T₃      | ![Diagram](image4) |
| BT₄       | ![Diagram](image5) |
| B₄T₄      | ![Diagram](image6) |
| OA₂       | ![Diagram](image7) |
| O₂A₂      | ![Diagram](image8) |
| OA₃       | ![Diagram](image9) |
| O₃A₃      | ![Diagram](image10) |
analyzed as doubly charged ions. For this reason, the drift times for the hexamers and octamers are lower than for the tetramers. Although the drift time differences between SDOs of equal chain lengths were very small, they turned out to be significant. This was shown by previous standalone measurements, where the SDOs were directly infused into the ESI-IMS-TOF mass spectrometer (Table S2). As a consequence, most of the analyzed SDOs can be identified by its drift time. This also applies for SDOs that only differ in their monomer sequence. Even SDOs of the same chemical structure showing different tacticity could be separated by IMS. Obviously, spatial arrangement of the side groups has an effect on the ion size, and thus on the drift time. A similar observation was recently reported by Imamura et al. for poly(diethylacrylamide) homopolymers containing different numbers of meso diads in the polymer chain.\[45\] In the present work, as expected, the influence of tacticity decreased by reducing the chain length, so that the single chiral monomers \(S-C\) and \(R-C\) could not be separated by IMS.

Since the drift time of an ion in the IMS cell depends on instrumental parameters (wave velocity, wave height, collision gas flow, etc.), the drift times were converted to collision cross sections (CCS) using a polyalanine calibration curve (Figures S1 and S2) in order to obtain a library that should be more comparable to other IMS systems (Figure 1(b), Table S1). The drift time is needed to determine the corresponding CCS values. Therefore, the calibration measurements were done with the same IMS measurement parameters as the drift time determinations of the SDOs. The CCS values allow the direct observation of structure information. As expected, the octamers showed the highest CCS values, followed by the hexamers (Figure 1(b), red box) and tetramers (Figure 1(b), blue box). The single monomers exhibited the lowest CCS values.

In the next step, to show up the true potential of the UHPLC-ESI-IMS hyphenation, mixtures of different SDOs were prepared and analyzed. The UHPLC was run in a size-exclusion mode, so that the oligomers were separated according to their hydrodynamic volume. The combination of the UHPLC separation columns was chosen to achieve a separation range of 200–5000 g/mol matching the preferred ionization window of the Synapt G2 HDMS mass spectrometer. Table 3 shows the components of each mixture. The mixture Mix A consists of samples with the same monomer units and different chain length. The mixture Mix B, on the other hand, consists of SDOs exhibiting all side chain functionalities at different chain lengths, thereby representing a more complex mixture.
Due to the prior fractionation of the oligomer mixtures, an elution profile for each component of the mixture could be extracted from the total ion current. Thus, the drift time distribution and the corresponding CCS values could be determined for each component of the mixture. Identification of the single components was achieved by comparing the CCS values obtained by analysis of the mixture to those listed in the previously created library. The UHPLC chromatograms for the mixtures are shown in Figures S3 and S4.

Figure 1. Drift times obtained by the analysis of SDOs using the hyphenation of UHPLC and ESI-IMS mass spectrometry (a); CCS values of all analyzed SDOs determined by using a polyalanine calibration (b), the tetramer samples were investigated in the singly charged states, the hexamer and octamer samples were investigated in the doubly charged state.

Due to the prior fractionation of the oligomer mixtures, an elution profile for each component of the mixture could be extracted from the total ion current. Thus, the drift time distribution and the corresponding CCS values could be determined for each component of the mixture. Identification of the single components was achieved by comparing the CCS values obtained by analysis of the mixture to those listed in the previously created library. The UHPLC chromatograms for the mixtures are shown in Figures S3 and S4.

The first mixture, Mix A, consists of three samples with different chain lengths. They are composed of the same two monomer units and vary between four and eight monomer units per chain. Although, the different chain lengths should result in different hydrodynamic volumes, the refractive index detector trace did not show separate peaks indicating the single components in
the mixture. However, the single oligomer elution profiles extracted from the total ion current exhibit a clear shift corresponding to the chain length (Figure 2(a)). Thus, size-exclusion separation did obviously occur.

Moreover, due to the extracted single oligomer elution profiles, mixture components showing a low ionizability could be detected. Although the mixtures were prepared with equal weight fractions of all components, the signal intensity of the samples is highly different. The tetramer shows an intensity of around $1 \cdot 10^2$ while the octamer shows an intensity of $2 \cdot 10^5$ (Figure 2(b)).

With the help of the obtained drift times (Figure 2(b), Table S3) the CCS values could be determined for the single mixture components. The identification of the single components was possible by comparison with the CCS reference values from the library (Table 4). The CCS deviation was significantly lower than 1% for the tetramer and hexamer, whereas the octamer showed a CCS deviation of 1.3%. With these results, sample B3T3 could be clearly identified. For the other two components a clear differentiation between block-wise and alternating monomer sequence would not be possible (BT2/B2T2 and BT4/B4T4).

Mixture Mix B contained tetramers bearing carboxylic acid, isooctyl and isoborneol side groups. Moreover, hexamers with carboxylic acid, benzylic, furfuryl, and isoborneol side groups were also present in this mixture. These multiple functionalities and small polymerization degrees represent a challenge for the initial chromatographic separation in size-exclusion mode. Nevertheless, the trace of the RI detector showed 3 distinct chromatographic peaks (Figure S4). A broad peak occurred between 1.4 and 1.8 min, followed by two overlapping peaks between 2.1 and 2.5 min. The different functionalities in mixture B also led to difficulties in the mass spectrometric separation. All 4 components required different parameters in the ESI ion source in order to obtain optimal signal intensities. When analyzing the mixture, a compromise for the ESI parameters had to be found. The extracted single oligomer elution profiles revealed that the first broad peak belonged to the CA3 (Figure 3(a)). The tetramer with carboxylic acid side groups (C2A2) also showed a rather broad single oligomer elution profile. These broad elution profiles

![Figure 2. Single oligomer elution profiles (a) and drift times (b) of the extracted components in the mixture Mix A.](image-url)
strongly indicate that these samples did not elute in a pure size-exclusion mode. Such enthalpic interactions were not surprising because the porous gel matrix in the separation columns comprised ethylene bridged silica particles, whose surface might interact with carboxyl acids via hydrogen bonding. Additionally, the single intensities for these two SDOs were very small, probably due to the non-optimal ionization parameters (Figure 3(b)). Comparing the obtained CCS values of the samples CA₃ and C₂A₂ to those in the library revealed that a clear identification of these compounds only with the help of CCS values was not possible (Table 5). The relative difference of up to 1.72% (for sample CA₃) is too large for an identification beyond doubt. However, the combined analysis of CCS values and m/z ratios allowed the clear distinction of the SDOs, except for monomer sequence and tacticity (Figure S5).

The two overlapping peaks of high intensity in both the chromatographic trace and the single oligomer elution profile could be assigned to the samples BT₃ and OA₂ (Figure 3, Table 5).

Table 4. CCS values of the components in mixture Mix A compared to the reference of individual measurements.

| Component | CCS_{Reference} (Å²) | CCS_{Mix} (Å²) | Difference (%) |
|-----------|----------------------|----------------|----------------|
| BT₂       | 374.46               | 375.40         | 0.25           |
| BT₃       | 474.58               | 474.58         | 1.01*10^-4     |
| B₄T₄      | 507.95               | 514.53         | 1.30           |

Figure 3. Single oligomer elution profiles (a) and drift times (b) of the extracted components in the mixture Mix B.

Table 5. CCS values of the components in mixture Mix B compared to the reference of individual measurements.

| Component | CCS_{Reference} (Å²) | CCS_{Mix} (Å²) | Difference (%) |
|-----------|----------------------|----------------|----------------|
| C₂A₂      | 374.90               | 374.57         | 0.09           |
| OA₂       | 370.15               | 369.43         | 0.19           |
| CA₃       | 467.72               | 459.68         | 1.72           |
| BT₃       | 475.65               | 475.42         | 0.05           |
A distinction between block-wise and alternating monomer sequence for sample OA$_2$ was not easily possible, whereas sample BT$_3$ could be clearly identified.

For a distinction between alternating and block-wise sequence of side chain functionalities, the difference of CCS values between measurement and reference obviously needs to be 0.05% or less. Signal intensity seems to have an influence on the deviation of CCS values between measurement and reference. Ionization parameters affecting signal intensity strongly depend on the chemical composition of the SDOs. Considering a complex mixture of different SDOs, finding the proper ionization parameters as well as avoiding ion suppression effects are crucial issues. Chromatographic separation provided the opportunity of infusing sample fractions to the ESI-IMS-TOF mass spectrometer. In future, a shift from size-exclusion mode to adsorption mode should provide a better separation according chemical composition of SDOs. As a consequence, optimization of ionization parameters for each sample fraction would be possible.

**Conclusion**

In this work, sequence-defined oligomers (SDOs) comprising different polymerization degrees, an alternating or block-wise sequence of side chain functionalities and different tacticities were analyzed using a hyphenation of UHPLC and ESI-IMS-TOF mass spectrometry. Ionization and ion mobility separation parameters were optimized with respect to a high signal intensity and large drift time difference. Each SDO showed an individual drift time, even allowing the distinction of SDOs with different monomer sequences and tacticity. After converting the instrument and measurement-specific drift times to more general collision cross section (CCS) values, a library was obtained for the set of analyzed SDOs. Subsequently, two different mixtures of SDOs were analyzed in order to identify the single components. The chromatographic separation prior to ESI-IMS-TOF analysis allowed the extraction of elution profiles for each SDO out of the total ion current. Thus, due to the higher sensitivity of the mass spectrometer, mixture components that could not be seen in the RI detector trace could be identified in the MS elution profile. In complex mixtures, SDOs with different chemical composition could be identified by using the CCS values in combination with the m/z ratios. A distinction between alternating and block-wise sequence of side chain functionalities was achieved for two SDOs in complex mixtures. So, hyphenation of UHPLC and ESI-IMS-TOF mass spectrometry proved to be a valuable tool for analyzing SDOs with functionalities and structures.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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