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2D-LC/SEC-(MALDI-TOF)-MS Characterization of Symmetric and Nonsymmetric Biocompatible PEO_m–PIB–PEO_n Block Copolymers

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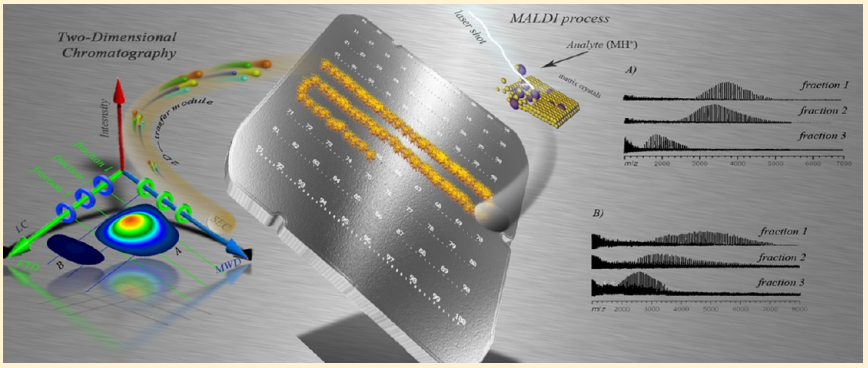
Supporting Information



ABSTRACT: Complex copolymer mixtures can be directly analyzed via multidimensional chromatographic techniques after successful synthesis. High-performance liquid chromatography coupled to size exclusion chromatography (LC/SEC) revealed detailed information on the chemical composition, polymeric structure, and molar mass distribution of copolymer mixtures, in particular of symmetric and nonsymmetric α-TEO-ω-PEO telechelic PIB copolymers. A series of azide/alkyne "click" reactions after living polymerization reactions were used to prepare the either symmetric or nonsymmetric PIB–PEO-based triblock copolymers of the general structure (PEO_n–PIB–PEO_m BCPs (with n = 3; m = 3, 12, or 17)). In order to demonstrate the efficiency of the "click" reaction and thus the purity of the final triblock copolymers, the critical conditions of the PIB-homopolymers (M_n = 3–30 kg mol^{-1}) in the isocratic elution mode (LCCC) were investigated. Thus, it was possible to separate the final polymers from their intermediates using a reversed-phase Atlantis-RP C18 column as stationary phase and a mixture of methyl-*tert*-butyl ether/methanol (85.34/14.66 (w/w)) as mobile phase. On the basis of the PEO segment length and overall hydrophobicity of the BCPs, we observed a complete separation of the stepwise "click" products. Finally, direct coupling of the 2D-LC/SEC to (MALDI-TOF) mass spectrometric techniques allowed a clear identification of all reaction steps proving the structure of the final symmetric and nonsymmetric triblock copolymers.

INTRODUCTION

Biomaterials, most of all those based on poly(ethylene oxide),[1−3] represent an enormously important class of polymers in pharmaceutical applications as they can selectively control the release and circulation of drugs via different principles such as diffusion[4] or water penetration[5] or via chemically controlled delivery[5] after appropriate response.[6] Therefore, changing the chemistry of the polymeric materials, e.g., introducing polyanhydrides,[7] polyesters,[8] poly(acrylic acid)s,[9] poly(methyl methacrylate),[10] and polyurethanes,[11] can enable a selective release of the active agent/drug by shielding the carrier sterically via closely attached PEO brushes from early cellular recognition and thus renal clearance.[3,12] This involves hydrophilic, amorphous, and low molecular weight polymers carrying functional side groups, in particular, biodegradable and biocompatible block copolymers based on their diversity of recent pharmaceutical applications.[13−15] Of particular interest are amphiphilic BCPs[16] as they can self-assemble into polymersomes[17−26] with a defined membrane thickness, enabling to address drug-delivery systems similar to conventional liposomes, but with significantly higher stability and drug loading.[27−35]

As the complexity of macromolecules (e.g., block copolymers) increases by the presently large plethora of synthetic approaches, more specific and sophisticated analytical methods are required to prove their exact structure, most of all with respect to their architecture, the attached end groups, and the exact chemical composition. The sole use of separation

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techniques based on the hydrodynamic volume of the polymer (e.g., size exclusion chromatography[36] (SEC)), which differ significantly from other separation techniques relying on chemical interactions, often does not enable the specific analysis (chemical structure and architecture) of structurally more complex polymers. Therefore, a considerable number of chromatographic techniques have been developed,[37] allowing to address the generation of a profile including the chemical composition distribution (CCD), the nature of functional side chains together with the molar mass distribution (MMD), the microstructure, and the underlying polymeric architecture. Many of these structural features are based on the exploitation of distinct adsorption effects, which are normally excluded in the SEC but required in liquid adsorption chromatography (LAC). The discovery of liquid chromatography of synthetic polymers under critical conditions (LCCC)[38−41] in particular allows the separation of block copolymers from their homopolymer precursors based solely on their different functionalities by eliminating the effects of their molecular weight.[42−50] In liquid chromatography, the main separation modes are governed by the interaction parameter c, which describes the interaction of the single (polymer) chain with the stationary phase.[51] Whereas in size exclusion chromatography (SEC) molecules are excluded from the pores in consequence of their hydrodynamic volume (being driven by entropy changes), chromatography in the adsorption mode (LAC) is driven by enthalpic changes of the structural units with the stationary phase, whose distribution coefficient is much larger than one, thus putting $K > 1$ (see eq 1).

$$\ln K = \Delta G = \Delta H - T\Delta S \quad (1)$$

The critical adsorption point (CAP), where all structural units of the polymer coelute together with the initial solvent regardless of their chain length, is given by the compensation of enthalpic and entropic driving forces. By addressing a careful match among the solvent composition, the respective polymer, and the polarity of the used separation matrix, the distribution coefficient is set to $K = 1$ and the interaction parameter $c = 0$,[52−55] to achieve the conditions required for a size independent, but polarity dependent, analysis of the polymers. In the analysis of di- and triblock copolymers (A_nB_m or $A_nB_mA_n$ types), the chromatographic separation method is usually based on the critical condition of the homopolymer A (LCCC) leading to an elution of a block copolymer now depending on the exact nature of its interaction with the stationary phase—either in the exclusion (SEC) or the adsorption mode (LAC). Starting from the well-known LCCC conditions of PEO[56]- and PMMA[57]-based polymers, a large variety of different A_nB_m block copolymers (e.g., poly(ethylene oxide)-*b*-poly(propylene oxide),[58] polystyrene-*b*-poly(ethylene oxide),[59] and polystyrene-*b*-poly(methyl methacrylate)[60]) have been analyzed using either one- or two-dimensional chromatography,[47,48,61−64] where the block composition varies strongly in their polarities.[65] Especially, the direct coupling of one- and two-dimensional chromatographic methods to mass spectrometry (MALDI and ESI)[66−71] has enabled to effectively combine the separation of BCPs based on functionality and hydrodynamic volume (SEC) into an online two-dimensional system, where an orthogonality criterion is fulfilled via distinctive separation mechanisms.[72] Thus, fractions collected and separated in the second dimension (SEC) can be directly split into a MALDI-TOF MS via a sample transfer module or into a ESI-TOF MS by direct online coupling, where the mass spectra are recorded and correlated with the two-dimensional fractions.[68]

In this article, we would like to highlight the multidimensional chromatographic analysis of either symmetric triblock copolymers (A_nBA_n) (tri(ethylene oxide)-*b*-polyisobutylene-*b*-tri(ethylene oxide) (TEO-PIB-TEO (**1a**)) or nonsymmetric triblock copolymers (A_nBA_m) poly(ethylene oxide)-*b*-polyisobutylene-*b*-tri(ethylene oxide); PEO_m−PIB−PEO_n (**1b**, **1c**). These and structurally related block copolymers can form either block copolymer micelles or polymersomal membranes[73] in dependence of their PIB/PEO ratio, which in turn is controlled via living polymerization methods. Both blocks (the PIB and the PEO) are biocompatible polymers, thus being prospective polymers for drug-delivery purposes. The applied synthetic methodology follows a combination of living carbocationic polymerization (LCCP) coupled to "click" chemistry[74−77] as an efficient tool to covalently link two different polymer chains. Use of the initiator methylstyrene epoxide in the LCCP of isobutylene specifically enables to prepare block copolymers with either a symmetric or nonsymmetric composition.[78] Subsequently, the stepwise "click" reaction has been monitored using 2D-LCCC/SEC coupled to MS (MALDI-TOF MS) to follow the reaction pathways and analyze the exact chemical structure of the obtained block copolymers. The purpose of this article is to demonstrate the distinctive role of multidimensional chromatographic methods coupled to mass spectrometric techniques to analyze the purity and the structural arrangement of symmetric or nonsymmetric BCPs.

EXPERIMENTAL PART

Materials. All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) and were used as received unless otherwise stated. All solvents, which were used for the work-up procedures, were distilled prior to use. Tetrahydrofuran (THF) and toluene were predried over potassium hydroxide for several days, refluxed over sodium/benzophenone under an argon atmosphere, and distilled. Methylstyrene epoxide (MSE) initiator was synthesized by the epoxidation reaction of methylstyrene with pure 3-chloro-1-peroxybenzoic acid (MCPBA) in dichloromethane (CH_2Cl_2) according to ref 79. α-Methoxy-ω-azido-telechelic triethylene oxide (**4**) was prepared in a two-step reaction starting via mesylation of the hydroxyl end group followed by conversion into the azido-telechelic compound via nucleophilic substitution using sodium azide (see Supporting Information), as reported in the literature.[80] The preparation of α-methoxy-ω-alkyne-telechelic triethylene oxide (**7**) or α-methoxy-ω-alkyne-telechelic poly(ethylene oxides) (**8**, **9**) was performed via phase transfer catalysis (see procedure, Supporting Information) according to a representative procedure by Teodorescu et al.[81,82] Bisallyl-telechelic polyisobutylenes, which were used to determine the LCCC conditions with M_n = 3000 (M_w/M_n = 1.2), 9000 (M_w/M_n = 1.1), and 29 300 g mol^{-1} (M_w/M_n = 1.2) were synthesized via a living carbocationic polymerization (LCCP) method using the bivalent initiator 1-*tert*-butyl-3,5-bis(1-chloro-1-methylethyl)benzene (DCCl).[83]

Synthesis. *Synthesis of α-TEO-ω-azido-telechelic PIBs (6).* According to the literature,[84] the synthesis of α-TEO-ω-azido-telechelic PIBs (**6**) was conducted by starting with the polymerization of isobutylene using MSE/$TiCl_4$ as initiator system, followed by a stepwise end-group modification as shown in the Supporting Information (procedures of all steps). The obtained product was dried under high vacuum yielding 87% of **6**. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.47 (s, 1H), 6.82 (d, J = 8.41 Hz, 2H), 4.64−4.46 (m, 2H), 4.08 (t, J = 5.78 Hz, 2H), 3.86 (t, J = 4.76 Hz, 1H), 3.72−3.47 (m, 12H), 3.37 (s, 3H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) 154.4 (C18), 158.8, 145.4, 142.8, 128.8, 125.7, 125.1, 120.9, 114.2,

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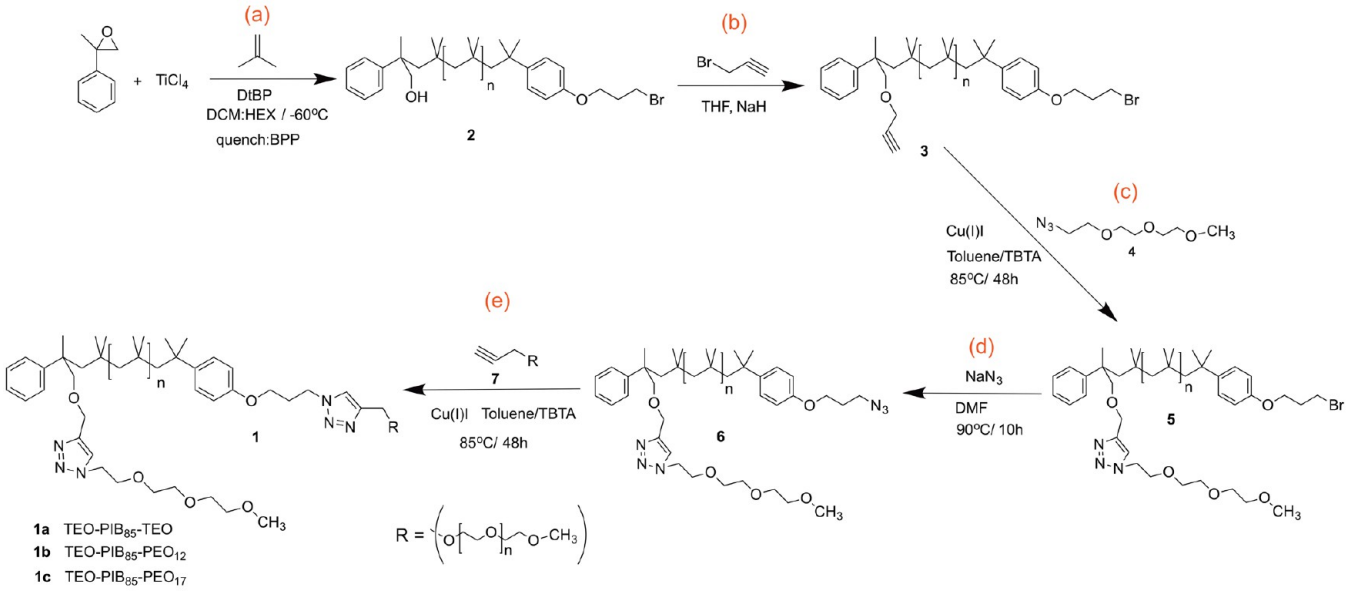
Scheme 1. Synthetic Pathway toward α-TEO-ω-PEO Telechelic PIBs Using Double "Click" Reactions via Cu(I) Toluene/TBTA at 85 °C



81.3, 71.8, 70.6, 69.8, 64.8, 63.5, 59.3, 52.9, 44.3, 43.1, 29.3, 27.6. FT-IR (KBr): ν (cm^{-1}) = 3000−2700 (C−H), ∼2100 (N_3).

Synthesis of Symmetrical or Nonsymmetrical α-TEO-ω-PEO-telechelic PIBs (1). The azide/alkyne "click" reaction between α-TEO-ω-azido-telechelic PIB (**6**) and α-methoxy-ω-alkyne-telechelic poly(ethylene oxides) (**7**, **8**, or **9**) varying in the number of repeating units was conducted under Cu^I-mediated conditions. Compound **6** (1 equiv), alkyne-telechelic triethylene oxide or poly(ethylene oxide) (1.1 equiv), TBTA (0.1 equiv), and Cu(I) iodide were dissolved in toluene. The reaction mixture was bubbled with argon for 1 h and then heated to 80 °C. After 48 h, the solvent was removed via rotary evaporation, and the crude product was purified by column chromatography on silica gel ($CHCl_3/CH_3OH$ = 30:1, R_f = 0.1 to remove the unreacted alkyne-telechelic PEO) yielding symmetrical or nonsymmetrical α-TEO-ω-PEO-telechelic PIB (**1**). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.59 (s, 1H), 7.47 (s, 1H), 6.82 (d, J = 8.41 Hz, 2H), 4.64−4.45 (m, 8H), 4.01 (t, J = 5.78 Hz, 2H), 3.86 (t, J = 4.76 Hz, 2H), 3.72−3.46 (m, (4m + 12)H), 3.37 (s, 6H), 2.38 (m, 2H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) 154.4, 158.8, 145.4, 142.8, 128.8, 125.7, 125.1, 121, 114.2, 81.3, 71.8, 69.8, 64.8, 63.5, 59.7, 52.9, 48.3, 43.1, 29.3, 27.8.

Spectroscopic Methods. *Nuclear Magnetic Resonance (NMR) Spectroscopy.* ^{1}H and ^{13}C NMR spectra were performed on a Varian Gemini 2000 (400 MHz) FTNMR spectrometer using MestRec-C (4.9.9.6) software for data interpretation. The measurements were done in deuterated chloroform ($CDCl_3$: 7.26 ppm (1H) and 77.0 ppm (^{13}C)). All chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethyl silane (TMS); coupling constants (J) are given in hertz (Hz) using standard abbreviations (s = singlet; d = doublet; t = triplet; m = multiplet).

Infrared (IR) Spectroscopy. IR spectra were recorded on a Bruker Vertex 70 FT-IR spectrometer and evaluated by OPUS (6.5) software. Samples were measured with a golden gate unit combined with a RT DLa TGS detector.

Chromatographic Methods. *Size Exclusion Chromatography (SEC).* SEC experiments were performed on a Viscotek GPCmax VE2001 system combined with a Viscotek TDA30L (triple detector array). Polyisobutylene standards in the range of 340−87 600 g mol^{-1} purchased from PSS (Polymer standard service) were used for conventional external calibration using a Viscotek VE3580 refractive index detector. The polystyrene−divinylbenzene based column set consists of a HHR-HGuard-17369 precolumn followed by a GMHHR-N-Mixed Bed 18055 (1000−4 × 10^5 g mol^{-1}), and a G2500HHR-17354 (100−2 × 10^4 g mol^{-1}) column. The detector and the column temperature were held constant at 35 °C with a flow rate of 1 mL/min. The investigated samples were dissolved in THF (>99.9%); analyses of the results of the GPC experiments were achieved using OmniSec (4.5.6) software.

High Performance Liquid Chromatography (HPLC). Measurements were performed on a LaChrom Elite by Hitachi VWR equipped with an autosampler, a highly accurate liquid delivery pump at a semimicro flow rate (maximum 2.4 mL/min), a degasser, a diode array detector (DAD) operating at 190−900 nm, and a column oven with temperature control (temperature set limit = 0−70 °C).

The separation of the block copolymers was carried out on a reversed phase Waters Atlantis-RP C18 column, 100 Å, 5 μm, dimension 4.6 × 250 mm. Methyl-*tert*-butyl ether (MTBE) and tetrahydrofuran (THF) were applied as the mobile phase system. The critical condition of bisallyl-telechelic polyisobutylene which further applied to LC/SEC experiments of the block copolymers was found at MTBE/MeOH = 14.66/85.34 (w/w) on RP-C18, at a temperature of 30 °C. Temperatures were maintained constant (±0.2 °C) throughout all experiments, and the injected sample volume was 20 μL. The DAD signals were recorded on EZchrom elite software version 3.3.2 SP2 with an operating wavelength from 190 to 900 nm at a sampling width of 800 ms to obtain sufficient data points across peaks.

Spectrometric Methods. *MALDI-TOF Mass Spectrometry.* MALDI-TOF MS measurements were performed on a Bruker Autoflex III system (Bruker Daltonics) operating in reflectron and linear modes. Data evaluation was carried out on flexAnalysis software (3.4). Ions were formed by laser desorption (smart beam laser at 355, 532, 808, and 1064 ± 5 nm; 3 ns pulse width; up to 2500 Hz repetition rate), accelerated by a voltage of 20 kV and detected as positive ions. The matrix solution was prepared by dissolving 1,8,9-anthracenetriol (dithranol) or *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) in THF at a concentration of 20 mg/mL. Polymer samples were dissolved in THF at a concentration of 20 mg/mL. AgTFA, NaTFA, and LiTFA were dissolved at a concentration of 10 mg/mL in THF. The solutions of the matrix, the polymer, and the salt were mixed in a volume ratio of 100:50:4, and 1 μL of each mixture was spotted on the MALDI target. The instrument was externally calibrated with a 25 poly(ethylene glycol) (PEG) standard (M_p = 2000 or 4000 g mol^{-1}) applying a quadratic calibration method with an error of 1−2 ppm.

Coupling Techniques. *Two-Dimensional Chromatography (LC/SEC).* The outlet of the HPLC (first dimension) is connected to the second dimension using an electronically controlled eight-port

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Table 1. Characterization of the Final Triblock Copolymers 1a−1d and Their Precursors 2c, 3, 5, and 6 Using Size Exclusion Chromatography (SEC), Nuclear Magnetic Resonance (NMR), and Their LC Retention Times with Different Flow Rates (0.1 and 0.3 mL/min)

		characterization						
entry	sample	M_n[a] (GPC)	M_w[a] (GPC)	PDI	M_n[a] (NMR)	chain length[b]	R_{t1}[c]/R_{t2}[d]	yield[f] [%]
1	HO-PIB_{85}-Br (**2c**)	4760	5980	1.25	5080	PIB_{85}	–[e]	95
2	alkyne-PIB_{85}-Br (**3**)	4920	6200	1.26	5160	PIB_{85}	7.13/31.0	90
3	TEO-PIB_{85}-Br (**5**)	4840	6100	1.26	5360	TEO-PIB_{85}	–[e]	82
4	TEO-PIB_{85}-N_3 (**6**)	4780	5920	1.24	5300	TEO-PIB_{85}	6.71/29.4	87
5	TEO-PIB_{85}-TEO (**1a**)	5280	6490	1.23	5500	TEO-PIB_{85}-TEO	6.33/27.1	78
6	TEO-PIB_{85}-PEO (**1b**)	5030	6240	1.24	5930	TEO-PIB_{85}-PEO_{12}	6.31/27.0	82
7	TEO-PIB_{85}-PEO (**1c**)	4540	5720	1.26	6150	TEO-PIB_{85}-PEO_{17}	6.31/27.0	67
8	TEO-PIB_{57}-TEO (**1d**)	4080	5220	1.28	3960	TEO-PIB_{57}-TEO	6.35/27.1	80

[a]Number or weight-average molar mass of the BCPs in g mol^{-1}. [b]Calculated number of the single polymer building blocks by NMR integration. [c]HPLC retention time (R_{t1}) of the investigated samples with 0.3 mL/min flow rate. [d]HPLC retention time (R_{t2}) of the investigated samples with 0.1 mL/min flow rate used in the 2D-HPLC/SEC setup. [e]Sample was not measured via HPLC or 2D-LC/SEC. [f]Isolated mass after precipitation and purification via silica gel column chromatography.

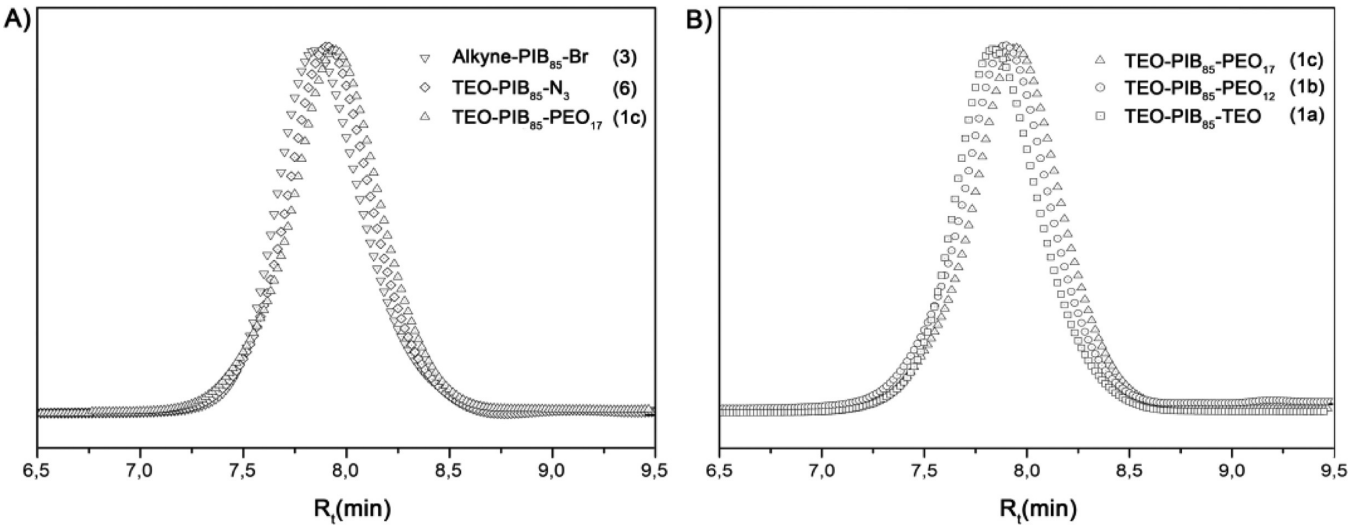

Figure 1. SEC traces of (A) the α-alkyne-ω-bromo-telechelic PIB (**3**), the α-TEO-ω-azido-telechelic PIB (**6**) obtained after the first "click" reaction and (B) the final triblock copolymers (**1a−c**) after the second "click" reaction (see also results in Table 1).

switching valve system (EPC8W, VICI Valco instruments, Houston, TX), with (2 × 200 μL) loops. The second dimension consisted of an LC-Pump (Viskotek/Malvern instruments) and an evaporative light scattering detector (ELSD) 300S Softa (SofTA Corporation, Broomfield, CO) for the detection. Nitrogen was used as carrier gas, the pressure of the nebulizer was set to 5.0 bar, and the evaporative temperature was set to 118 °C. For size exclusion separation, a PSS PFG high-speed column with the size 50 × 20 mm (Polymer Standard Services, PSS, Mainz, Germany), 300 Å, and a particle size 5 μm was used. All measurements in the second dimension were performed using HPLC-grade THF as the mobile phase running at a flow rate of 5 mL/min for one-dimensional GPC/SEC. Data acquisition was performed using PSS UDC 810 Universal Data Center; evaluation and extraction of data were accomplished using PSS WinGPC Software.

Two-Dimensional Chromatography (LC/SEC) Coupled to MALDI-TOF MS. Further investigation of functionality type distribution (FTD) by means of MALDI-TOF MS was achieved. The sample fractions were continuously conveyed to the TM-sprayer (Leap Technologies, Carrboro, NC) by splitting the flow subsequent to PFG column with a microvolume cross-connector (Cat. no. MX1CS6, Valco Instruments, Houston, TX), fitting size 1/16 in. DCTB (20 mg/mL) was used as MALDI matrix and sodium trifluoroacetate (1 mg/mL) as cationizing reagent. DCTB and NaTFA were premixed in a 50:1 v/v ratio (matrix:salt) and transferred using an isocratic pump with a flow rate of 2 mL/min. To achieve a uniform and consistent coating, the nozzle height was set to 8 cm, nitrogen was used as a carrier gas with a flow rate of 5 L/min, and the fractions were deposited on MALDI-target with a spray speed of 9 mm/min at 130 °C in a spray linear mode. The spraying process was monitored using LEAP Technologies control software.

RESULTS AND DISCUSSION

Synthesis of Symmetric and Nonsymmetric Tri-BCPs. The synthetic route toward the stepwise construction of the symmetric (α-TEO-ω-TEO block copolymers **1a**) and the nonsymmetric α-TEO-ω-PEO block copolymers **1b** and **1c** is based on methods that have been developed earlier in our group.[84] Using methylstyrene epoxide as initiator for the living cationic polymerization (see Table S1, Supporting Information) followed by azide/alkyne "click" chemistry,[85] the functionalization of the α,ω-end group of the PIBs with different moieties is enabled (see NMR characterization, Supporting Information) as illustrated in Scheme 1. Basically, the residual hydroxy moiety of the initiator moiety allows for an orthogonal functionalization of both polymer chain ends of the intermediate (**2**), as the transformation to the alkyne-PIB_{85}-Br (**3**) now enables the separate attachment of PEO chains of different chain lengths. The final BCPs **1a**, **1b**, and **1c** were

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thereby synthesized via successive stepwise "click" chemistry by attaching first a polar triethylene glycol (TEO) unit on the α-end group followed by a TEO or PEO unit onto the ω-end group in high yields with complete end-group efficiency as judged by excessive NMR spectroscopy (see Supporting Information). MALDI investigations and GPC are shown in entries 5−7 of Table 1. The purpose of this article is to demonstrate the distinctive role of multidimensional chromatographic methods coupled to mass spectrometric techniques to analyze the purity and the structural arrangement of the final BCPs together with their synthetic presteps.

One-Dimensional Liquid Chromatography (SEC and HPLC). The polyisobutylenes bearing the alkyne end group (alkyne-PIB$_{85}$-Br (**3**)) and the products with the attached hydrophilic moieties, TEO-PIB$_{85}$-N$_3$ (**6**) (as diblock copolymer) and TEO-PIB$_{85}$-PEO$_n$ (**1b**, **1c**) as triblock copolymers, were examined using conventional size exclusion chromatography (calibrated against PIB standards). In Figure 1, the GPC traces of TEO-PIB$_{85}$-N$_3$ (**6**) as well as the alkyne-functionalized polyisobutylene precursor α-alkyne-ω-bromo-telechelic PIB (**3**) and the final triblock copolymers (**1a−c**) are displayed. The peak maximum of the triblock copolymer (**1c**) is slightly shifted toward higher retention volume (TEO-PIB$_{85}$-PEO$_{17}$ (**1c**), R_t = 7.96 min) compared to the retention volume of the diblock copolymer (TEO-PIB$_{85}$−N$_3$ (**6**), R_t = 7.92 min) and the alkyne-functionalized polyisobutylene (alkyne-PIB$_{85}$-Br (**3**), R_t = 7.84 min); see Figure 1A.

However, a small shift in the opposite direction can be distinguished by varying the chain length of the PEO block in the triblock copolymers **1a−c**, as illustrated in Figure 1B (TEO-PIB$_{85}$-TEO (**1a**), R_t = 7.83 min) and (TEO-PIB$_{85}$-PEO$_{12}$ (**1b**), R_t = 7.91 min). The shift in the peak maxima displays a significant change toward lower molar mass values M_n, although the molecular weight is increasing. Experimental GPC results of a series of poly(ethylene oxides) samples varying their chain lengths and architecture showed that longer PEO chains may lead to reduced hydrodynamic radii due to their limited solubility in THF.[86] Consequently, with increasing the PEO block length in tri-BCPs (**1a** to **1c**), the retention time of the BCPs on the SEC column increases caused by the fact that THF is a poor solvent for the attached PEO blocks, resulting in a more contracted chain conformation (lower hydrodynamic radius).

In order to enable two-dimensional liquid chromatography as a tool for the characterization of the "click" reaction steps toward the synthesis of the BCPs, the symmetric and nonsymmetric α-TEO-ω-PEO telechelic PIBs **1a−c** were inspected via LC/SEC, and the final products were successfully fingerprinted by MALDI-TOF MS. As a first step toward a successful LCCC chromatography, the critical conditions of polyisobutylene homopolymers (PIB) were investigated, using bisallyl-telechelic polyisobutylenes as model-systems (M_n = 3000 (M_w/M_n = 1.2), 9000 (M_w/M_n = 1.1), and 29 300 g mol^{-1} (M_w/M_n = 1.2)) on a reversed phase column (Atlantis-RP C18). The practical approach in which LCCC is projected for polyisobutylene starts with a thermodynamically good solvent such as methyl-*tert*-butyl ether (MTBE), where the homopolymer would behave in the exclusion mode and the molar mass decrease is noticed by the shift to higher retention volumes, as illustrated in Figure 2.

Upon the addition of a nonsolvent such as methanol to the main elution mobile phase (methyl-*tert*-butyl ether) up to 10.56% (w/w), the interaction of the PIB homopolymer with the stationary phase increases, resulting in raised enthalpic interactions, which however still is insufficient to eliminate the entropic effect. Nevertheless, by further increasing the amount of the nonsolvent to 19.56% (w/w), we observed the adsorption chromatography mode (LAC). By fine-tuning the solvent mixtures, the critical conditions of the bisallyl-telechelic-polyisobutylene could be found at the composition of MTBE/MeOH, 84.4/14.6 (w/w) (see Figure 2).

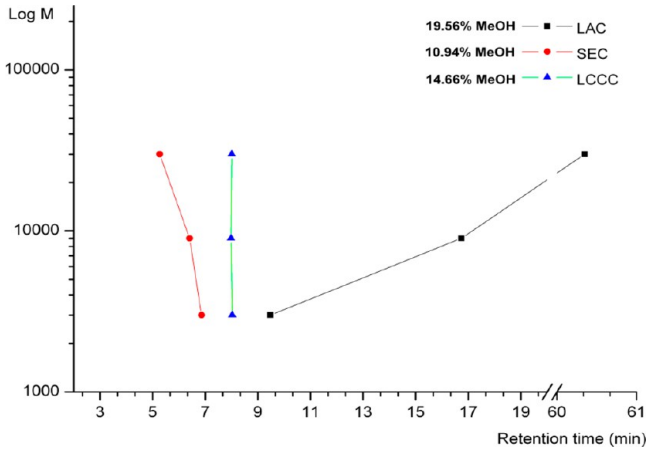

Figure 2. Plot of log M_n vs retention time (R_t) for the PIB-homopolymers (bisallyl-telechelic polyisobutylenes (M_n = 3−30 kg mol^{-1})), which demonstrate their retention behavior at constant temperature (30 °C) using a reversed-phase column (Atlantis-RP C18) as stationary phase. By varying the composition of the mobile phase (MTBE/MeOH), different chromatographic interaction modes of the polyisobutylene samples were obtained.

After setting up the critical conditions (LCCC) of PIB using a reversed-phase column (Atlantis-RP C18), poly(ethylene oxide) would plainly display an entropic behavior and therefore is expected to exhibit separation in the SEC mode. Hence, increasing the hydrophilicity (PEO$_n$, from n = 3 to 17) would allow the α-TEO-ω-PEO telechelic PIBs (**1a−c**) to elute faster. Consequently, the PIB-precursors **3** and **6** as well as the triblock copolymers (**1a−c**) synthesized via stepwise "click" reactions could now be investigated under the obtained critical conditions (see Figure 3). Accordingly, chromatographic traces of the precursor alkyne-functionalized polyisobutylene (**3**), TEO-PIB$_{85}$-N$_3$ di-BCP (**6**), and TEO-PIB$_{85}$-TEO triblock copolymer (**1a**) were recorded individually under LCCC. As shown in Figure 3A, increasing the hydrophilic composition by adding up one polar moiety (TEO) allowed the TEO-PIB$_{85}$-N$_3$ diblock copolymer (**6**) to elute faster (R_t = 6.71 min) than the alkyne-functionalized polyisobutylene (**3**) which eluted at R_t = 7.13 min. Correspondently, increasing further the hydrophilicity of the BCPs by attaching an additional poly(ethylene oxide) unit via a second "click" reaction allowed the triblock copolymer (**1a**) to elute significantly earlier (R_t = 6.33 min) compared to its precursors due to the reduced interaction between the polymer molecules and the hydrophobic stationary phase. Ipso facto, an excellent separation of the PIB-polymers **1a**, **3**, and **6** under the given conditions could be achieved.

By attaching longer chains of PEO (n = 12 or 17) to the ω-end of the triblock copolymer **1b** and **1c**, we expected that a shift of the elution volume toward lower retention times should be observed caused by the further increase in the polymer hydrophilicity. However, the increase of the PEO unit up to n = 17 showed no noticeable shift of the BCP retention times, as

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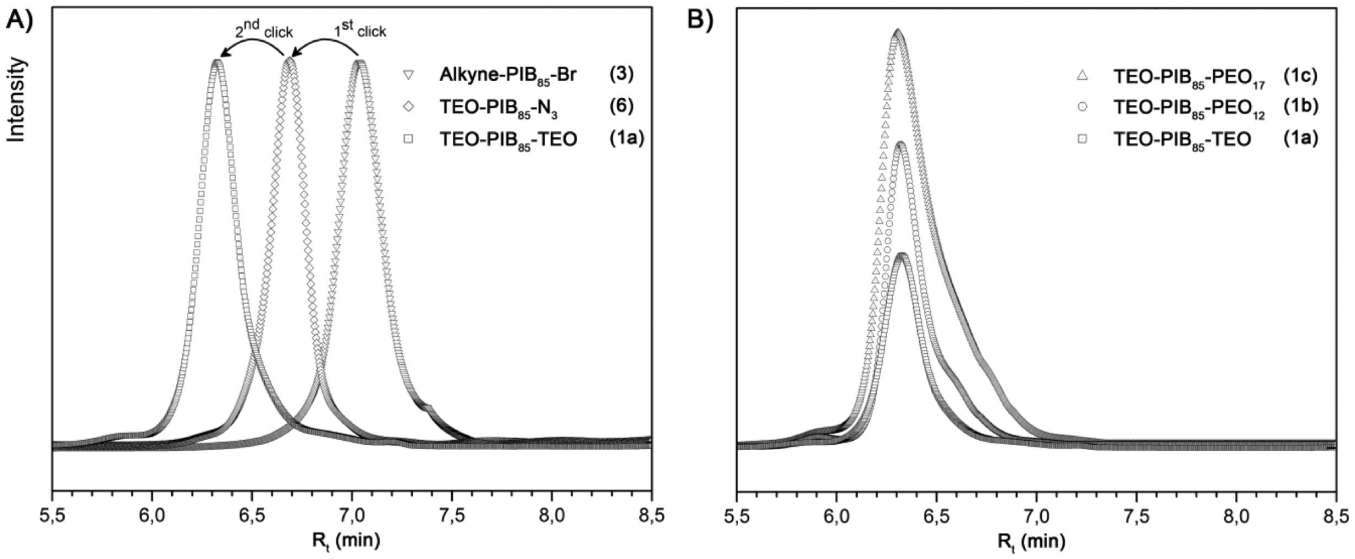

Figure 3. LC traces, measured at critical conditions of PIB at a flow rate of 0.3 mL/min, T = 30 °C of (A) α-alkyne-ω-bromo-telechelic PIB (**3**), α-TEO-ω-azido-telechelic PIB (**6**) obtained after the first "click" reaction, and the final triblock copolymer (**1a**) after the second "click" reaction demonstrating a stepwise shift to lower retention times (R_t) with increasing their hydrophilicity by attaching polar PEO blocks. (B) The final triblock copolymer (**1a**–**c**) shows the same main R_t with peak broadening to higher R_t by upon increasing the PEO block length.

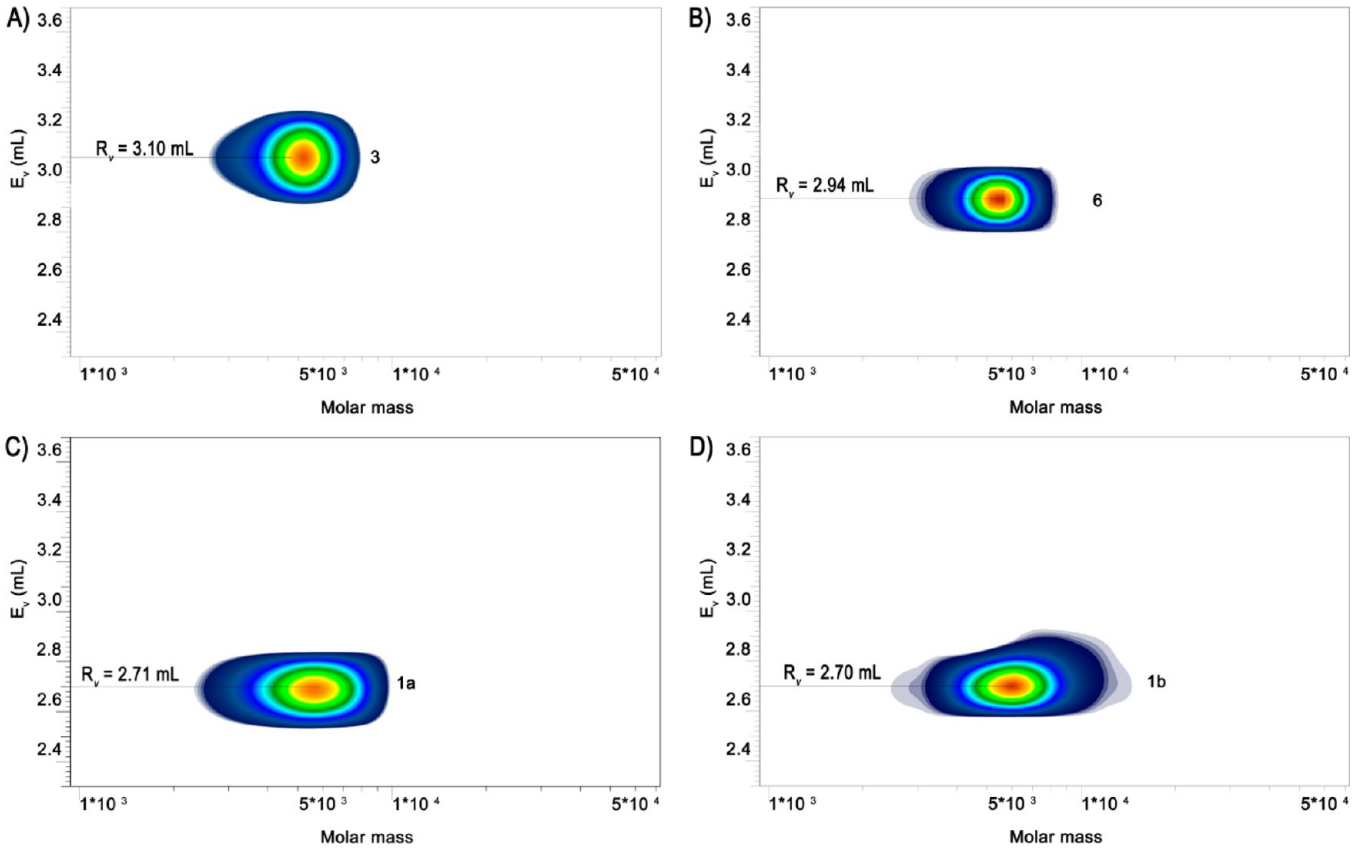

Figure 4. 2D-LC/SEC plots of (A) the α-alkyne-ω-bromo-telechelic PIB (**3**) used as starting material, (B) the TEO-ω-azido-telechelic PIB (**6**) obtained after first "click" reaction, and (C, D) the final triblock copolymer (**1a** and **1b**) after the second "click" reaction measured under LCCC for PIB in MTBE/MeOH using a weight percent mixture of 85.34/14.66.

listed in Table 1. In Figure 3B, it is significant to notice that the full width at half-maximum (fwhm) elution peak in the case of the triblock copolymers **1b** and **1c** increases significantly in comparison to BCP **1a**, due to the additional EO units causing a broader polydispersity. It turns out that a direct discrimination between the symmetric and nonsymmetric tri-BCPs can only be achieved if a supplemental set of information from the mass of the molecules is enabled. Therefore, two-dimensional chromatography was coupled to mass spectrometry to identify the triblock copolymers in question.

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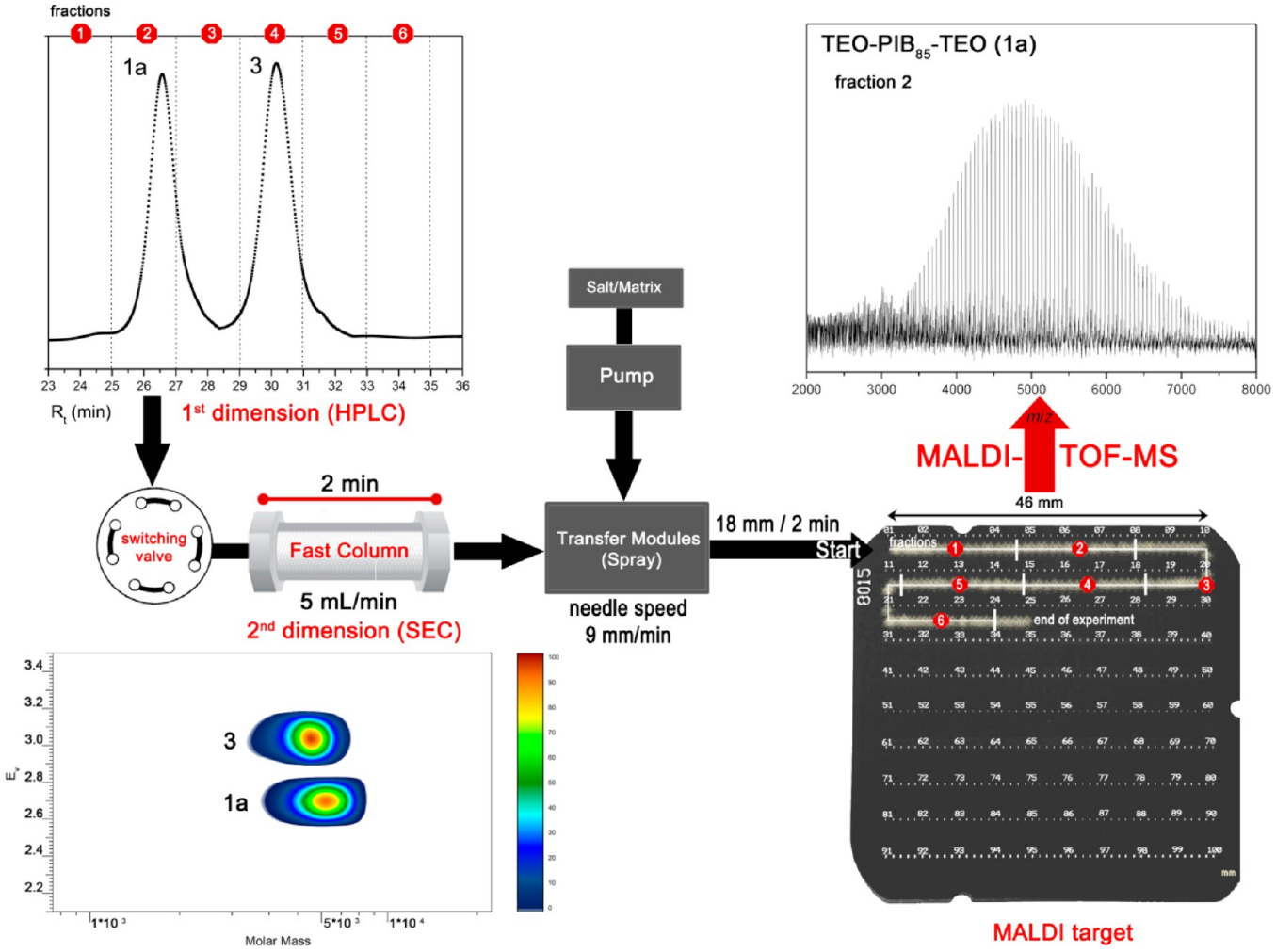

Figure 5. Schematic representation of the used chromatographic setup (2D-LC/SEC) coupled to MALDI-TOF mass spectrometry via a transfer modulus sprayer, demonstrated for a mixture of compound **3** and the BCP **1a**. Separation of the compounds takes place in the first dimension (HPLC), and after collecting different fractions using a collector/switching valve the second dimension (SEC) is measured. Coupling to MALDI-TOF via transfer modulus allowed a continuous investigation of each fraction at various elution volumes.

Two-Dimensional Liquid Chromatography Measurements (2D-LC/SEC). In a first step, the successful transformation of α-alkyne-ω-bromo-telechelic PIB (M_n = 4920 g mol^{-1}) (**3**) to α-TEO-ω-azido-telechelic PIB (**6**) can be monitored by the 2D setup (LC/SEC) using the previously developed critical conditions of the bisallyl-telechelic PIBs. The 2D contour plot (see Figure 4A, B) shows no detectable amounts of alkyne-residues (R_t = 31 min, see Table 1) in compound **6**, which elutes at (R_t = 29 min) after extensive purification. Additional 2D measurements were conducted before the purification step (see synthetic part), which allowed to detect that approximately 8 wt % residual of compound **3** (see Figure S8) were present in the initially nonpurified product. This again demonstrates the high efficiency of the 2D setup (LC/SEC).

In the second "click" reaction, an additional polar moiety (PEO with various chain lengths, n = 3−17) was attached on the ω-position of the α-TEO-ω-azido-telechelic PIB (**6**) which increased the hydrophilicity of the polymer molecules leading again to a shift to earlier retention times of the final triblock copolymers ($R_t \sim$ 27 min, see Table 1). In the 2D contour plots of the BCPs (**1a**, **b**, Figure 4C, D), the results of the 2D separation of tri-BCPs as presented in Figure 4 are shown, indicating no noticeable shift in the 2D contour diagram when increasing the EO units of the tri-BCPs from 3 to 12 units. Even by further increasing the polarity of 12 and 17 units of PEO were insufficient to distinguish the symmetric tri-BCP (**1a**) in the first dimension (LC, see Figure 3B) from the nonsymmetric tri-BCPs (**1b** and **1c**). Therefore, a direct coupling after the second dimension (SEC) with MALDI-TOF MS to distinguish between the final chemical structures of different BCPs was essential.

Two-Dimensional Chromatography Coupled to Mass Spectrometry (2D-LC/SEC-MALDI-TOF MS). The 2D-LC/SEC setup only offered a separation in the case of the polymers displaying different polarities (e.g., polymers **3** and **6**), thus depicting the reaction efficiency and purity together with the molar mass distribution (MMD). Therefore, the complexity of the modification of the symmetric (**1a**) and nonsymmetric triblock copolymers (**1b**, **1c**) demanded more complex analytical methods based on mass spectrometry techniques.

Thus, a setup for the coupling of 2D-LC/SEC toward MALDI-TOF was developed, which should allow a direct separation in two dimensions (size and polarity) and a detection of the individual mass spectrum of the separated polymers. In addition, an extensive information on the purity of the obtained products is envisioned and thus of significant

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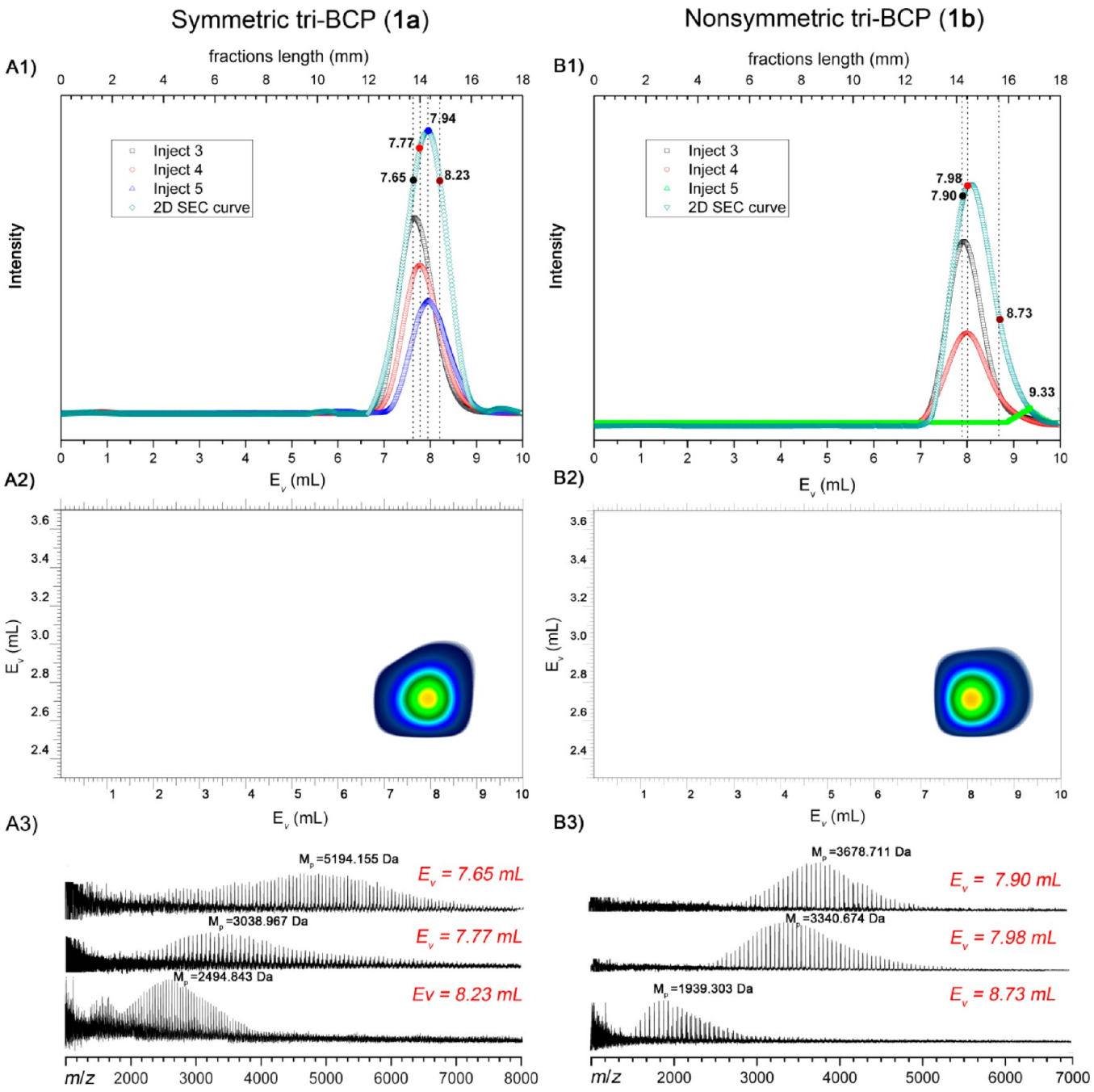

Figure 6. 2D-LC/SEC measurements of the symmetric tri-BCP **1a** (A1−A3) and the nonsymmetric tri-BCP **1b** (B1−B3) coupled to MALDI-TOF MS allowing the continuous monitoring of the chemical structure at different elution volumes (E_v): (A1, B1) recorded 2D-SEC curves with the single SEC curves of each injected fraction, (A2, B2) the corresponding 2D-LC/SEC plots, and (A3, B3) the obtained MALDI-TOF spectra at different E_v proving the final chemical structures.

importance. Therefore, the setup of two-dimensional chromatography is also used as a tool to depict the efficiency and the purity of all reaction steps. Consequently, in the first step, a mixture of the alkyne-telechelic polyisobutylene (**3**) and α,ω-TEO-telechelic PIB (**1a**) was analyzed as demonstrated in Figure 5 to investigate the separation and, in consequence, prove the final structure of the block copolymers. Based on the critical conditions of the PIB-homopolymers (3−30 kg mol^{-1}), the LC trace of the mixture (**1a** and **3**) as shown in Figure 5 represents the number of fractions calculated for one dimension. Subsequently, the loop volume (0.2 mL × 2 loops) connected to the switching valve allows each fraction to continuously transfer into a high-speed SEC column running at a flow rate of 5 mL/min. Therefore, molecules are separated based on their hydrodynamic volume, and their average molar mass M_n can be calculated. Furthermore, each fraction eluting from the high-speed column has a retention time of 2 min (E_v = 10 mL), which can be consequently conveyed to a sample transfer module (TM sprayer). The upcoming stream is continuously mixed with a mixture of NaTFA/DCTB (20 mg/mL) and further sprayed on a MALDI target, where each fraction has a defined track length (46 mm). Therefore, taking advantage of an automated experiment, one can predetermine the number of required tracks that would cover the number of

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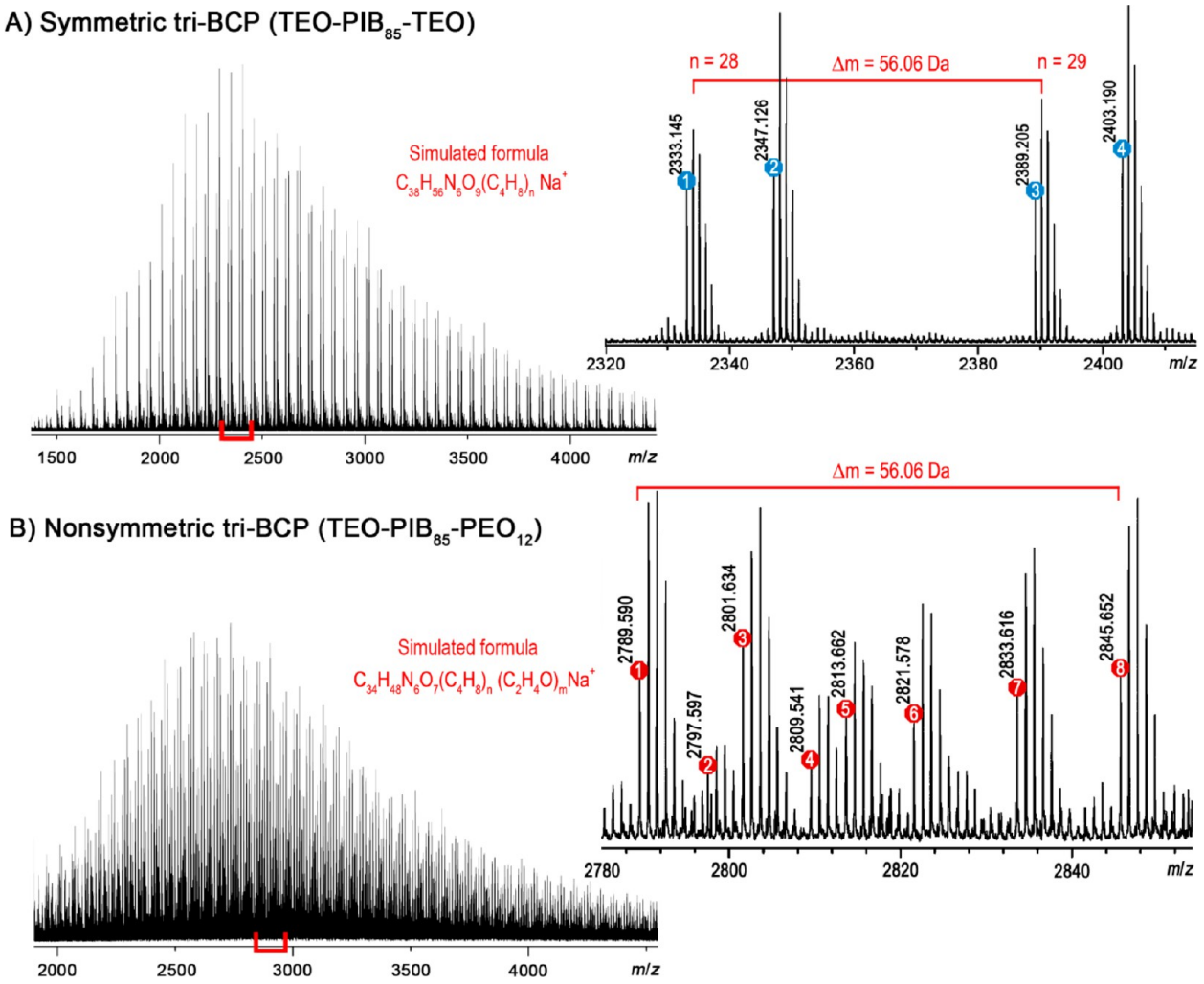

Figure 7. MALDI-TOF MS characterization of the symmetric BCP **1a** (A) and the nonsymmetric BCP **1b** (B) recorded after separation in the second dimension (2D-LC/SEC-MALDI-TOF MS). The obtained MALDI-TOF spectra showing different m/z series with $\Delta m/z$ = 56.06 g mol^{-1}, which were successfully identified and assigned to the final copolymer structures (see Tables 2 and 3), proving the chemical composition and purity of the investigated samples.

fractions of the second dimension. Hence, we were able to directly correlate the recorded MS spectra with the previous SEC fractions.

After drying, the MALDI target was inserted into a MALDI-TOF MS to identify the chemical composition and the final structure of the respective polymers, as matrix-assisted laser desorption ionization−time-of-flight mass (MALDI-TOF MS) offers a high selectivity and allows desorption of a large variety of molecules. The evaluation of the molecular mass provided by MALDI-TOF-MS does not serve to usefully analyze synthetic polymers with a broader molecular weight distribution. Therefore, a fractionation of highly polydisperse polymers via size exclusion chromatography (SEC), thus providing a narrow molar mass distribution (MMW) for each fraction, opens the possibility to combine the information collected by two-dimensional chromatography and mass spectrometry (MALDI-TOF MS) in a useful manner. As shown in Figure 6, fractions collected from the second dimension (SEC) are further analyzed in depth by MALDI-TOF MS as a mass detection method leading to a continues monitoring of the chemical structure at each elution volume.

Figures 6A1 and 6B1 represent single SEC curves recorded from different injections (collected fractions) using an evaporative light scattering detector (ELSD) directly coupled to the SEC. The 2D curve renders the overall graphical representation of the molar mass extracted from the intensities of all injections achieved throughout the 2D-measurements. The 2D contour plot (see Figures 6A2 and 6B2) of the symmetric (**1a**) and nonsymmetric (**1b**) tri-BCPs is then directly correlated to the 2D SEC curves. Each injection and its corresponding fraction are continuously sprayed onto a MALDI-target via a sprayer transfer module (TM sprayer). The run time of one single SEC measurement takes 2 min (E_v = 10 mL) and thus represents a 18 mm fraction length on the MALDI-target. To correlate the monitored masses at each specific elution volume with a chemical structure that proves the desired molecular architecture of the investigated samples, MALDI-TOF MS was performed as depicted in Figure 6A3 for the symmetric tri-BCP (**1a**) and in Figure 6B3 for the nonsymmetric polymer (**1b**). The monitored masses by MALDI-TOF MS with increasing elution volume confirmed the SEC behavior of the analyzed BCPs.

The recorded MALDI-TOF spectra in Figure 7 unveiled the functionality type distribution (FTD) of the block copolymers in question. The symmetry of the BCP **1a** (α,ω-TEO-telechelic PIB) in Figure 7A is depicted by the assignment of the main

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Table 2. Experimental Results of 2D-LC/SEC Offline Coupled to MALDI-TOF MS of Symmetric Tri-BCP (1a)

entry	polymer structure[a]	species	m/z found [g mol^{-1}]	m/z simulated [g mol^{-1}]	error [ppm]
1	n = 28	$[C_{150}H_{280}N_6O_9]$ Na^+	2333.145	2333.153	6.1
2	n = 29[b]	$[C_{151}H_{282}N_6O_8]$ K^+	2347.126	2347.148	9.3
3	n = 29	$[C_{154}H_{288}N_6O_9]$ Na^+	2389.205	2389.216	6.0
4	n = 30[b]	$[C_{155}H_{290}N_6O_8]$ K^+	2403.190	2403.210	8.4

[a] $\sum = C_{38}H_{56}N_6O_9(C_4H_8)_n$. [b] Fragmentation ($-C_2H_4OCH_3$).

Table 3. Experimental Results of 2D-LC/SEC Offline Coupled to MALDI-TOF MS of Nonsymmetric Tri-BCP (1b)

entry	polymer structure[a]	species	m/z found [g mol^{-1}]	m/z simulated [g mol^{-1}]	error [ppm]
1	n = 33; m = 6	$[C_{178}H_{336}N_6O_{13}]$ Na^+	2789.590	2789.571	7.0
2	n = 30; m = 10	$[C_{174}H_{328}N_6O_{17}]$ Na^+	2797.597	2797.488	6.5
3	n = 34; m = 5	$[C_{180}H_{340}N_6O_{12}]$ Na^+	2801.634	2801.607	9.7
4	n = 31; m = 9	$[C_{176}H_{332}N_6O_{16}]$ Na^+	2809.541	2809.524	5.9
5	n = 35; m = 4	$[C_{182}H_{344}N_6O_{11}]$ Na^+	2813.662	2813.644	6.6
6	n = 32; m = 8	$[C_{178}H_{336}N_6O_{15}]$ Na^+	2821.578	2821.561	6.3
7	n = 33; m = 7	$[C_{180}H_{340}N_6O_{14}]$ Na^+	2833.616	2833.597	6.6
8	n = 34; m = 6	$[C_{182}H_{344}N_6O_{13}]$ Na^+	2845.652	2845.633	6.5

[a] $\sum = C_{34}H_{48}N_6O_7(C_4H_8)_n(C_2H_4O)_m$.

species $[C_{38}H_{56}N_6O_9(C_4H_8)_{28}]Na^+$; the monoisotopic peak found m/z = 2333.145 g mol^{-1} agrees with the simulated structure m/z = 2333.153 g mol^{-1} with an error of 6.1 ppm (see Table 2). The species $[C_{151}H_{282}N_6O_8]$ K^+ (m/z = 2347.126 g mol^{-1}) as observed in MALDI-TOF MS spectra could be assigned to the main peak fragmentation of the last $-(C_2H_4OCH_3)$ unit. Even though a potassium salt was not intentionally added as cationization agent, the potassium ion associated with homologues distribution could still be observed as contamination in glasswares and solvents due to the fact that potassium hydroxide was applied to predry solvents which were used in the reported synthetic pathway, similar to reports by other authors investigating PEO-polymers via MALDI-TOF MS.[87,88] Furthermore, the delayed ion extraction technique enables more time for cation attachment based on the affinities of polymer samples.[87]

The nonsymmetric BCP (α-TEO-ω-PEO-telechelic PIB **1b**) was also analyzed in 2D-LC/SEC-MALDI-TOF MS. The additional PEO unit, which exhibits a polydispersity compared to the TEO chain end, led to a much more complex MALDI spectrum, showing several side series characterized by the variation of PEO units (4−10), as shown in Table 3.

The chain length distribution (CLD) extracted from Figure 7B shows a main series with six PEO units. Hence, the main monoisotopic peak, m/z = 2789.590 g mol^{-1}, corresponds to the structure $[C_{34}H_{48}N_6O_7(C_4H_8)_{33}(C_2H_4O)_6]Na^+$ which agrees with the simulated isotopic pattern (compare Table 3). Nevertheless, two different patterns would describe the PEO distribution in the triblock copolymer α-TEO-ω-PEO-telechelic PIB (**1b**): the decrease of the PEO unit (n = 6, 5, and 4) with increasing PIB chain, Figure 7B (labels 1, 3 and 5), and the other pattern which can be distinguished by increasing the content of the PEO (n = 6, 7, 8, 9, and 10) with decreasing in the PIB content, Figure 7B (labels 8, 7, 6, 4, and 2). The latter pattern shows a broader chain length distribution (CLD) characterized by a significantly reduced ionization efficiency (intensity) compared to the first pattern, in which a stronger and narrow distribution can be observed. Table 3 clearly demonstrates well-resolved isotopic patterns of the nonsymmetric triblock copolymer alongside of the PEO chain length distribution (CLD).

CONCLUSION

In this publication a direct coupling between two-dimensional LC/SEC under LCCC conditions with MALDI methods was investigated for the analysis of symmetric (**1a**) and nonsymmetric (**1b**, **1c**) PIB−PEO based triblock copolymers. A series of two stepwise azide/alkyne "click" reactions after living carbocationic polymerization based on methylstyrene epoxide as initiator was used to prepare the either symmetric (**1a**) or nonsymmetric (**1b**, **1c**) triblock copolymers (PEO_m−PIB−PEO_n BCPs (with m = 3; n = 3, 12, or 17)). Demonstration of the efficiency of the "click" reaction and the purity of the final triblock copolymers was accomplished by searching for the critical conditions of the PIB-homopolymers (M_n = 3−30 kg mol^{-1}) in the isocratic elution mode (LCCC). Using a reverse phase Atlantis-RP C18 column, it was possible to separate the final polymers from intermediate polymers via a mixture of methyl-*tert*-butyl ether/methanol (85.34/14.66 (w/w)) as the mobile phase. On the basis of the PEO segment length and the overall hydrophobicity of the tri-BCPs, we observed a complete separation of the stepwise "click" products. Final analysis of the different block copolymers together with the molecular weight was then accomplished by direct coupling of the 2D-LC/SEC to (MALDI-TOF) mass spectrometry via a spray module, which was directly connected after the 2D-LC/SEC system. This enabled to resolve the so-achieved separation into a series of time-correlated MALDI-spectra. A clear identification of the symmetric (**1a**) and nonsymmetric (**1b**, **1c**) triblock copolymers could be accomplished, additionally allowing to identify all prereaction steps and thus proving the structure and purity of the final symmetric and nonsymmetric triblock copolymers. The presented methodology allows to achieve a true picture of the purity of block copolymers after linkage or direct crossover reactions—an aspect which is important for use of these polymers as drug-delivery vesicles, most of all in the investigation of polymersomal membranes, formed from such or similar block copolymers.

ASSOCIATED CONTENT

Supporting Information

Synthesis of α-hydroxymethyl-ω-bromo telechelic PIB (**2**) (S1.1), experimental results of isobutylene polymerization

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(Table S1), synthesis of α-alkyne-ω-bromo-telechelic PIB (**3**) (S1.2), synthesis of α-TEO-ω-bromo-telechelic PIB (**5**) (S1.3), synthesis of α-TEO-ω-azido-telechelic PIB (**6**) (S1.4), synthesis of alkyne-telechelic PEOs (S1.5), synthesis of α-methoxy-ω-azido telechelic triethylene oxide (TEO) (**4**) (S1.6), ^{1}H NMR of α-hydroxyl-ω-bromo-telechelic polyisobutylene (**2**) (Figure S1), ^{1}H NMR of α-alkyne-ω-bromo-telechelic polyisobutylene (**3**) (Figure S2), ^{1}H NMR of α-TEO-ω-bromo-telechelic polyisobutylene (**5**) (Figure S3), ^{1}H NMR of α-TEO-ω-azido-telechelic polyisobutylene (**6**) (Figure S4), ^{1}H NMR of symmetric TEO-PIB$_{85}$-TEO block copolymer (**1a**) (Figure S5), ^{1}H NMR of nonsymmetric TEO-PIB$_{85}$−PEO$_{12}$ block copolymer (**1b**) (Figure S6), ^{1}H NMR of nonsymmetric TEO-PIB$_{85}$−PEO$_{17}$ block copolymer (**1c**) (Figure S7), 2D-LC/SEC spectrum of unpurified compound **6** after click reaction demonstrating same impurities of alkyne-telechelic PIB (3) (Figure S8), 2D-LC/SEC spectrum of symmetric TEO-PIB$_{57}$-TEO block copolymer (**1d**) (Figure S9), 2D-LC/SEC spectrum of nonsymmetric TEO-PIB$_{85}$-PEO$_{17}$ block copolymer (**1c**) (Figure S10). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

BCP, block copolymer; CLD, chain length distribution; DAD, diode array detector; ELSD, evaporative light scattering detector; FTD, functionality type distribution; LAC, liquid adsorption chromatography; LC, liquid chromatography; LCCC, liquid chromatography at critical conditions; LCCP, living carbocationic polymerization; MALDI-TOF MS, matrix assisted laser desorption ionization−time-of-flight−mass spectrometry; MeOH, methanol; MTBE, methyl-*tert*-butyl ether; MWD, molar weight distribution; M_n, number-average molar mass; PEO, poly(ethylene oxide); PIB, polyisobutylene; RI, refractometric detector; RP-C18, Atlantis reversed phase column; SEC, size exclusion chromatography; TEO, triethylene oxide; TM sprayer, transfer module sprayer; THF, tetrahydrofuran; 2D, two-dimensional chromatography; 2D LC/SEC-ESI-TOF-MS, liquid chromatography coupled in parallel to size exclusion chromatography and ESI-TOF-MS; 2D LC/SEC, LC coupled to SEC; 2D LC/SEC-(MALDI)-TOF MS, LC coupled in series to SEC and MALDI-TOF MS (via TM-sprayer).

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