

AQUEOUS SEC ANALYSIS OF CATIONIC POLYMERS AS GENE CARRIERS*

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Abstract Cationic polymers have been receiving much attention as non-viral gene vectors. The aqueous mobile phase was optimized in combination with Shodex OHpak SB columns for size-exclusion chromatography (SEC) analysis of disulfide-containing poly(ethylene imine) (PEI) derivatives used in gene delivery. Addition of acetonitrile in mobile phase was shown to be able to suppress the hydrophobic interactions between polymer analytes and the stationary phase. The absolute molecular weights and distributions of the cationic polymers were determined directly from online SEC-MALS (multi-angle light scattering)/RI (refractive index detector). The results demonstrate that a good SEC separation of disulfide-containing PEI derivatives used in gene delivery with little band broadening was achieved.

Keywords: PEI derivatives; Gene delivery; Aqueous SEC; Molecular weight; Online MALS detector.

INTRODUCTION

Water-soluble cationic polymers have been receiving much attention as non-viral gene carriers^[1], which condense plasmid DNA by ionic interactions. Among them, poly(ethylene imine) (PEI) has been regarded as the “golden” standard showing relatively high transfection efficiency, particularly in vitro, due to the proton sponge effect^[2, 3]. High molecular-weight (HMW) PEIs such as 25 kDa PEI are highly effective in gene transfection, but also induce high cytotoxicity due to the high cationic density and lack of biodegradability^[4]. Low molecular weight (LMW) PEIs, such as 1.8 kDa PEI, are less cytotoxic but poor efficient in gene transfection^[4, 5]. The molecular weight of the cationic polymer is one of the key characteristics, which can influence the transfection efficiency and the cytotoxicity of the polymer/DNA polyplexes^[6].

Size-exclusion chromatography (SEC), which is also referred to as gel-permeation chromatography (GPC), is a common analytical technique for the determination of molecular weight and molecular weight distribution (MWD) of polymers^[7]. However, aqueous SEC is tricky for the analysis of polyelectrolytes, due to the interaction between sample molecules and the packing material, which disturbs separation efficiency and reproducibility^[8]. Because almost all common column packings bear a negative surface charge, in particular SEC of cationic polymers is problematic^[9, 10]. There are many the so-called secondary effects, such as ion-exclusion (sample elutes early), ion-interaction and hydrophobic interactions (sample elutes late) which affect the chromatographic behavior of polyelectrolytes. Thus, salts are often added in the mobile phase to prevent polyelectrolytes chain expansion. However, when the salt concentration of the mobile phase is too high, hydrophobic interactions may occur^[9, 10]. Hydrophobic interactions can be minimized by addition of an organic

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solvent to the eluent^[10, 11]. The SEC conditions are not universal, optimization is usually required for a specific polymer.

Disulfide bonds can be reduced and cleaved by intracellular glutathione (GSH)^[12], and then induce dissociation of polymer/DNA complexes and releasing DNA to exhibit its function^[1, 13, 14]. Disulfide-containing PEI derivatives were synthesized by click chemistry^[15] for gene delivery in our group. The purpose of this study was to find the best SEC elution conditions for such PEI derivatives, and their absolute molecular weights and MWDs were determined using SEC-MALS (multi-angle light scattering).

EXPERIMENTAL

Materials

Branched poly(ethylene imine) (PEI) with molecular weight of 1.8 kDa was purchased from Alfa Aesar. Methanol and acetonitrile were supplied by Fisher Scientific (HPLC grade) and used without any further purification. The cationic polymers disulfide-containing polyethylenimine derivatives investigated in this work were synthesized by click chemistry, using CuBr as catalyst as described previously^[16, 17]. All other chemicals were analytical grade and used as received.

SEC-RI System

To optimize the aqueous SEC conditions, a Waters model 510 HPLC pump, a Spark Holland Basic Marathon autosampler with a loop volume of 20 μ L and a Shodex RI-101 refractive index detector (RI) were used to perform the SEC experiments. Only one Shodex OHpak SB-803 HQ column (300 $\times$ 8.0 mm) was used and the various mobile phases were investigated. The sodium acetate eluent (0.3 mol/L NaAc, pH 4.4) was prepared by dissolving a calculated amount of sodium acetate in reverse osmosis water, and the pH was adjusted to 4.4 with acetic acid. The eluents 0.3 mol/L NaAc (pH 4.4) with 30% methanol or 30% acetonitrile were made by mixing buffer 0.3 mol/L NaAc (pH 4.4) and organic solvents in *V/V* ratio. The obtained eluents were filtrated through a 0.22 μ m HPLC filter and degassed prior to use by ultrasound. The column was thermostated at 30°C and the flow rate was 0.5 mL/min.

SEC-Multiangle Light Scattering (MALS)/RI System

To determine the absolute molecular-weight and molecular-weight distribution (MWD) of the cationic polymers, we used an SEC-MALS/RI system consisted of a Waters 2690D separations module, a Waters 2414 RI detector and a Wyatt DAWN EOS MALS detector. The MALS detection measures the light-scattering intensity at 18 different angles and the RI detection measures the concentration of the polymer. This instrument contained a built-in autoinjector with a sample loop allowing injection of variable sample volumes. Two chromatographic columns (Shodex OHpak SB-803 and SB-802.5, Showa Denko, Japan) with a precolumn (Shodex SB-G) were used in series. The flow rate was 0.3 mL/min. The refractive index increment dn/dc value of PEI (1.8 kDa) was determined as 0.245 mL/mg from the RI response assuming sample mass recovery from the column of 100% and used for all the PEI derivatives in this work. The data collection and the data analysis were done with Astra software (Wyatt Technology).

RESULTS AND DISCUSSION

Synthesis of Disulfide-containing Polyethylenimine Derivatives by Click Chemistry for Nonviral Gene Delivery

Branched poly(ethylene imine) (PEI) with low molecular weight of 1.8 kDa was first functionalized by activated azidopropanol, 3-azidopropyl ester of carbonylimidazole. The resulting azide-terminated PEI was then cross-linked with disulfide-containing dialkyne BPPA-Cyst (bis[(propargyl carbamate)ethyl] disulfide) by click reaction as described previously^[16], to obtain a disulfide-containing reduction-sensitive PEI derivative with high molecular weight, denoted as PEI-SS-CL. The structure of the obtained soluble polymer PEI-SS-CL was confirmed by ¹H-NMR and FTIR^[16]. The structure illustration of the disulfide-containing PEI-SS-CL is shown in Fig. 1. However, the SEC elution behaviors of the obtained PEI-SS-CLs, combining Shodex SB columns (Shodex OHpak SB-803 and SB-802.5 with a precolumn Shodex SB-G) and 0.3 mol/L NaAc (pH 4.4) as the

mobile phase, was not reasonable. Tailing peaks of PEI-SS-CLs were observed, which partially overlapped with the solvent peak, and PEI-SS-CLs eluted surprisingly later than the branched PEI 1.8 kDa (data not shown). Another novel disulfide-containing brushed polyethylenimine derivative, denoted as P(Asp-Az-Al-ss-PEI)(Asp-EA)_x, was synthesized by click chemistry between mono-alkyne-terminated PEI (1.8 kDa) with disulfide linkage and poly(aspartic acid) with azide groups in side chains^[17]. Its schematic structure is shown in Fig. 2. Same problem was observed for the SEC analysis of such disulfide-containing brushed polyethylenimine P(Asp-Az-Al-ss-PEI)(Asp-EA)_x.

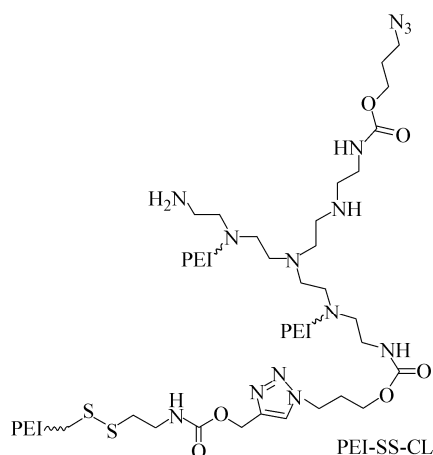


Fig. 1 Structure illustration of the disulfide-containing cross-linked PEI derivative PEI-SS-CL

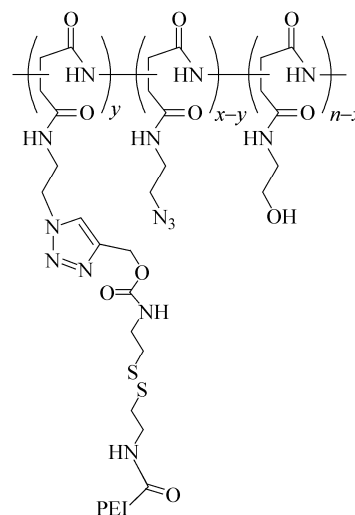


Fig. 2 Schematic structure of disulfide-containing brushed PEI derivative P(Asp-Az-Al-ss-PEI)(Asp-EA)_x

Optimization of the Aqueous Mobile Phase

In previous paper^[10] a suitable SEC method for molecular-weight characterization of various cationic gene-delivery polymers was established and applied for poly(2-(dimethylamino)ethyl methacrylate)s (PDMAEMAs), using 0.3 mol/L NaAc (pH 4.4) as the mobile phase and Shodex SB columns. It worked fine at the same SEC conditions for SEC analysis of branched PEI (25 kDa). It was also found that 0.3 mol/L NaAc (pH 4.4) is a suitable eluent in combination with Shodex OHpak SB columns for SEC analysis of brushed PDMAEMAs^[6]. However, the same SEC combination conditions were not suitable for the obtained PEI derivatives PEI-SS-CLs and P(Asp-Az-Al-ss-PEI)(Asp-EA)_x.

To optimize the aqueous SEC conditions, only one Shodex OHpak SB-803 column was used and the various mobile phases were investigated. As shown in Fig. 3, the PEI-SS-CLs, supposed to be much higher in molecular weight than the starting polymer branched PEI 1.8 kDa, came out later than the branched PEI 1.8 kDa, which indicated the presence of enthalpic interaction (adsorption) with the stationary phase when eluent 0.3 mol/L NaAc (pH 4.4) was used. We found in previous paper^[18] that 30% methanol added into the mobile phase of 0.3 mol/L NaAc, pH 4.4 with two PL aquagel-OH 30 columns (8 mm, 300 × 7.5 mm, Polymer Labs) could effectively suppress the enthalpic interactions of bio-reducible poly(amido amine)s with the stationary phase. As seen in Fig. 4, when eluent 0.3 mol/L NaAc (pH 4.4) with 30% methanol was used, the elution behavior of the PEI-SS-CLs, was improved and less tailing was observed. But still, the PEI-SS-CLs, supposed to be much higher in molecular weight than the starting polymer branched PEI 1.8 kDa, came out not earlier than the PEI 1.8 kDa, which suggests enthalpic interactions existing. When 30% acetonitrile was added to the mobile phase (0.3 mol/L NaAc, pH 4.4), as shown in Fig. 5, no tailing was observed and better separation was obtained for the PEI-SS-CLs. This indicates that the hydrophobic interactions existing between PEI-SS-CLs and the stationary phase can be suppressed by adding acetonitrile to the mobile phase.

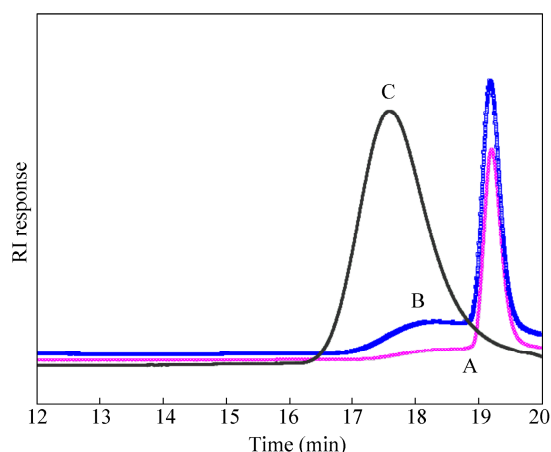


Fig. 3 SEC chromatograms of cationic polymers PEI-SS-CLs and 1.8-kDa-PEI in eluent 0.3 mol/L NaAc (pH 4.4) with flow rate of 0.5 mL/min: (A) PEI-SS-CL2, (B) PEI-SS-CL1, and (C) 1.8-kDa-PEI

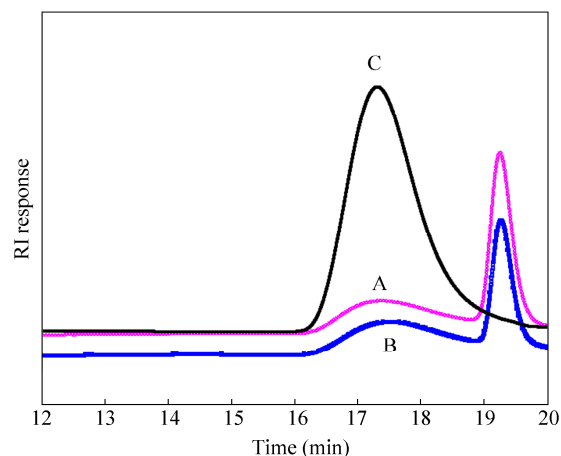


Fig. 4 SEC chromatograms of cationic polymers PEI-SS-CLs and 1.8-kDa-PEI in eluent 0.3 mol/L NaAc (pH 4.4) with 30% methanol with flow rate of 0.5 mL/min: (A) PEI-SS-CL2, (B) PEI-SS-CL1, and (C) 1.8-kDa-PEI

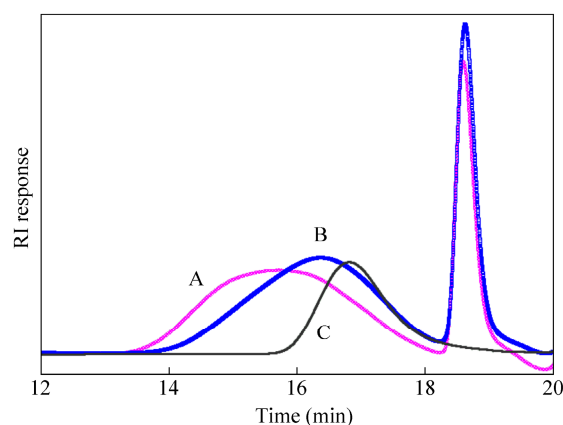


Fig. 5 SEC chromatograms of cationic polymers PEI-SS-CLs and 1.8-kDa-PEI in eluent 0.3 mol/L NaAc (pH 4.4) with 30% acetonitrile with flow rate of 0.5 mL/min: (A) PEI-SS-CL2, (B) PEI-SS-CL1, and (C) 1.8-kDa-PEI

Injected Amount (Volume) Effect on Polymer SEC Chromatogram

When a mixture of 70% 0.3 mol/L NaAc solution, pH 4.4 and 30% acetonitrile was used as the eluent, good separation was obtained for the SEC analysis of brushed $P(\text{Asp-Az-Al-ss-PEI})(\text{Asp-EA})_x$. Figure 6 shows the injected amount (volume) (with fixed polymer concentration) effect on the SEC chromatogram of the brushed $P(\text{Asp-Az-Al-ss-PEI})(\text{Asp-EA})_x$. The integrated area was proportional to injected volume (and thus amount) in the investigated range (0.10–1.0 mg), which indicates the absence of the strong enthalpic interaction (adsorption) between polymer and stationary phase. A similar effect of the injected amount on the SEC elution behavior was also observed for PEI-SS-CLs.

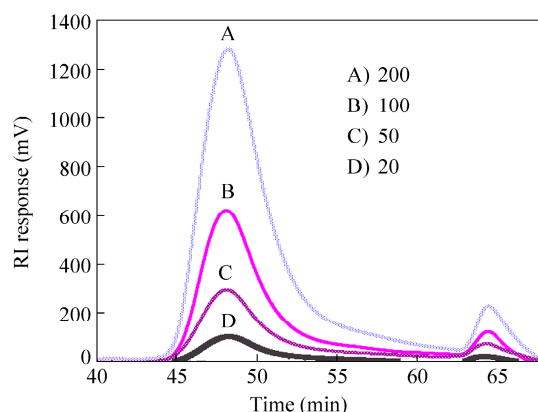


Fig. 6 Effect of the injected amount of brushed P(Asp-Az-Al-ss-PEI)(Asp-EA)_x on the SEC chromatograms Columns: Shodex OHPak SB-G, SB-803 and SB-802.5; Eluent: 0.3 mol/L NaAc (pH 4.4) with 30% acetonitrile; Flow rate: 0.3 mL/min; Polymer concentration: 4 mg/mL; The variation of the injected amount was realized by varying the injection volume from 20 μ L to 200 μ L indicated in the figure.

Molecular-weight and Molecular-weight Distribution of Cationic Polymers by SEC/MALS

Online coupling of a molecular-weight-sensitive MALS detector was carried out to determine the absolute molecular-weight and molecular-weight distribution (MWD) for cationic polymers and to verify the absence of secondary effects at the optimal aqueous SEC conditions. Figure 7 shows the SEC/MALS chromatograms of two polymers (a disulfide-containing PEI derivative PEI-SS-CL2 with high molecular weight and LMW 1.8-kDa PEI) and the corresponding absolute molecular-weight versus elution volume (*i.e.*, local calibration line). The low molecular-weight 1.8-kDa PEI had a lower light scattering (LS) response and a higher RI response, whereas the PEI derivative PEI-SS-CL2 with high molecular-weight displayed the reverse. This is reasonable because the LS detector is more sensitive to polymers with high molecular-weight. The two local calibration lines reasonably fit to one line over a wide molecular-weight range, indicating that a good SEC separation of PEI and its derivatives with little band broadening is obtained with the used eluent 0.3 mol/L NaAc, pH 4.4 with 30% acetonitrile in combination with the Shodex SB columns. The average values of molecular-weight and MWD can be obtained from the combined measurements of molecular-weight (weight average, MW) and the concentration obtained with the RI detector for each elution slice, as summarized in Table 1.

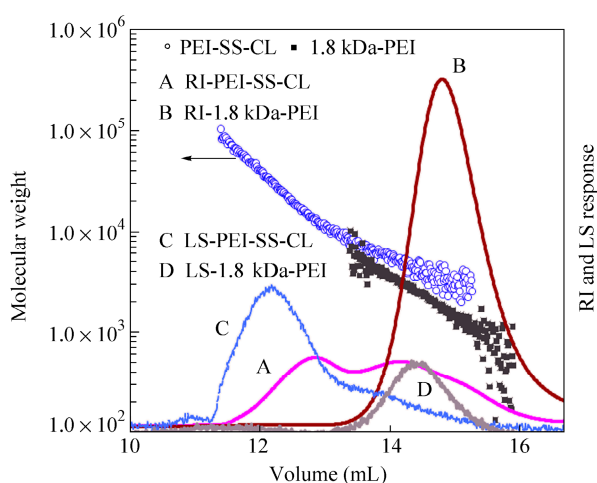


Fig. 7 The dependencies of molecular weight versus elution volume for PEI-SS-CL2 and 1.8 kDa PEI and the corresponding RI (A and B) and MALS (at 90°-angle, C and D) chromatograms

Table 1. SEC results of the disulfide-containing PEI-SS-CLs

Sample	M_n (kDa)	M_w (kDa)	PDI
PEI-SS-CL1	3.0	9.4	3.12
PEI-SS-CL2	7.7	14.2	1.85
PEI-SS-CL3	4.9	9.22	1.87
1.8 kDa PEI	1.49	2.06	1.38

CONCLUSIONS

The aqueous mobile phase was optimized in combination with Shodex OHpak SB columns for SEC analysis of disulfide-containing cross-linked PEI derivative PEI-SS-CL used in gene delivery, by adding acetonitrile to suppress the hydrophobic interactions between polymer analytes and the stationary phase. These conditions were also shown to be suitable for the disulfide-containing brushed polyethylenimine P(Asp-Az-Al-ss-PEI)(Asp-EA)_x. The absolute molecular weights and distributions of the cationic polymers were obtained directly from online SEC-MALS/RI. The results demonstrate that a good SEC separation of disulfide-containing PEI derivatives used in gene delivery with little band broadening was achieved.

Appendix: Some figures with essential colour discrimination can be found in the on-line version.

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