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Research Paper

Molar-Mass Characterization of Cationic Polymers for Gene Delivery by Aqueous Size-Exclusion Chromatography

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Purpose. This study was performed to develop a reliable aqueous size-exclusion chromatography (SEC) method to obtain the absolute molar masses and distributions of various cationic polymers used in gene delivery.
Methods. Water-soluble cationic [2-(dimethylamino) ethyl methacrylate] polymers (PDEs) with different molar masses and low polydispersities were synthesized by living polymerization and these were used to optimize the SEC conditions. Online coupled multiangle light scattering (MALS) detection was applied to obtain the absolute molar masses. Narrow fractions of high molar mass were obtained by semipreparative SEC.
Results. It was found that 0.3 M NaAc (pH 4.4) is a suitable eluent in combination with Shodex OHpak SB columns for SEC analysis of PDEs and other cationic polymers, such as poly(L-lysine) and poly(ethylene imine). The absolute molar masses of different PDEs were determined directly using SEC–MALS. A calibration curve was established using narrow PDEs.
Conclusions. A reliable routine method for molar-mass characterization of cationic polymers was established. Because standards of known molar masses with narrow distributions are not commercially available for most polymers used in pharmaceutics and biotechnology, the procedure described in this work can also be applied for molar-mass characterization of other water-soluble polymers.

KEY WORDS: gene delivery; molar mass distribution; online multiangle light scattering; SEC; water-soluble polymers.

INTRODUCTION

Water-soluble cationic polymers are under investigation as nonviral gene-transfection agents, which condense plasmid DNA by ionic interactions (1–3). Well-known examples of these polymers are poly(L-lysine) (PLL) (4), poly(ethylene imine) (PEI) (5), and poly(2-(dimethylamino) ethyl methacrylate) (PDE) (6,7). Besides these, other cationic polymers such as chitosan and its derivatives (8,9), polyphosphazenes (10), poly(alpha-(4-aminobutyl)-L-glycolic acid) (PAGA) (11), and the recently reported side-chain degradable poly(carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacryloylamino)-ethyl ester) (PHPMA-DMAE) (12), have been investigated for gene delivery purposes. The transfection efficiency and the cytotoxicity of cationic polymers complexed with plasmid DNA are strongly dependent on their molar masses, as reported for PDE (13,14), PLL (15), and PEI (16–19). In general, the cytotoxicity increases with increasing molar mass of the cationic polymer. Therefore, a reliable method for the characterization of molar mass and distribution of the cationic polymers is of critical importance.

The common analytical technique for the determination of molar mass of polymers and their distribution is size-exclusion chromatography (SEC), which is also referred to as gel-permeation chromatography (GPC) when chromatographic characterization of synthetic organic polymers is concerned or as gel-filtration chromatography (GFC) in case of aqueous solutions for water-soluble polymers or biopolymers (20). The molar mass averages and the molar mass distribution (MMD) can be obtained by using a calibration curve that relates the (logarithm of the) molar mass to the retention time or volume, or by using online molar mass detectors, such as light scattering or viscometry. The eluent in SEC should be strong enough to prevent enthalpic interactions of dissolved macromolecules with the stationary phase in the column. However, aqueous SEC is problematic for the analysis of polyelectrolytes due to their interactivity and the lack of suitable calibration standards (21–23). Because almost all common column packings bear a negative surface charge, in particular SEC of cationic polymers seems to be difficult (24–26). Volet and Lesec (26) reported that it

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is impossible to correctly characterize by SEC certain cationic polymers (such as copolymers of acrylamide and *N*,*N*,*N*-trimethylaminoethyl chloride acrylate with a cationicity of more than 20%). There are many 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. Ion exclusion and ion interaction can be suppressed by the addition of salt. However, when the ionic strength of the eluent is too high, hydrophobic interactions will occur (24,27). Hydrophobic interactions can be minimized by addition of an organic solvent to the eluent (28). Our group has extensively investigated the water-soluble cationic polymer PDE and its copolymers and analogues in gene delivery systems (6,13,14,29–32). Therefore, in this paper suitable eluent conditions of aqueous SEC of PDEs were first explored. The resulting conditions were also tested for other cationic polymers used in gene delivery.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich at the highest available purity and used as received, unless otherwise stated. 2,2′-Azobisisobutyronitrile (AIBN), 4,4′-azobis(4-cyanopentanoic acid), and 2-(dimethylamino)ethyl methacrylate (DMAEMA) were obtained from Fluka (Buchs Switzerland). Sodium acetate (NaAc) was from Merck (Darmstadt, Germany) and acetic acid (HAc) from Acros (Geel, Belgium). DMAEMA was distilled over calcium hydride prior to polymerization. Water purified by reverse osmosis was used throughout the study. Poly(ethylene glycol) (PEG) standards (with molar masses ranging from 400 to 996,000 Da) were purchased from Polymer Laboratories (Church Stretton, UK). Dextran standards (with molar masses ranging from 360 to 49,000,000 Da) were obtained from Fluka (Buchs, Switzerland). The cationic polymers used in this work are described next.

Synthesis of PDE by Anionic Polymerization

Low-molar-mass PDEs were synthesized via living anionic polymerization under dry nitrogen at −78°C (33). Diphenylmethyllithium was used as initiator in the presence of lithium chloride and tetrahydrofuran (THF) was the solvent. The concentration of DMAEMA was 0.5 M. The molar concentration of lithium chloride was ten times that of the initiator diphenylmethyllithium (monomer-to-initiator ratios were different, see Table I). After a certain time (Table I), the polymerization was terminated by adding methanol. ^{1}H NMR spectra were recorded on a Gemini 300-MHz spectrometer (Varian, Palo Alto, CA, USA) with relax delay of 2 s. The number-average molar mass (M_n) was calculated based on the ratio of the polymer protons at 4.1 ppm (2 H) to the initiator protons at 7.2 ppm (10 H).

Synthesis of PDE by Reversible Addition–Fragmentation–Chain Transfer Polymerization

Direct controlled polymerization of DMAEMA via the reversible addition–fragmentation–chain transfer (RAFT) process (34,35) was applied to synthesize high molar mass PDEs. 4-Cyanopentanoic acid dithiobenzoate, prepared following the literature (35,36), and 4,4′-azobis(4-cyanopentanoic acid) were used as the RAFT agent and initiator, respectively. Stock solutions of the RAFT agent and initiator in THF were used (molar ratio of 5:1). After evaporating THF in a nitrogen stream, DMAEMA dissolved in a HCl solution was added and the pH was adjusted to 5 with 4 M HCl (final monomer concentration 1.2 M). Polymerizations were done using different ratios of monomer to RAFT agent (Table II). The polymerizations were carried out under a nitrogen atmosphere at 70°C overnight. After cooling to room temperature, the polymer was poured into a dialysis tube (MWCO 3500), extensively dialyzed against water, and collected by lyophilization.

Table I. PDEs Synthesized by Living Anionic Polymerization

Sample	[Monomer]/[Initiator]	Time (min)	Conversion	$M_{n,th}$ (kDa)	M_n (kDa) (NMR)
PDE1	160	10	55	13.8	12.1
PDE2	160	25	73	18.3	15.7
PDE3	127	120	95	21.4	/
PDE4	160	100	99	24.8	23.4

$M_{n,th}$, theoretical number-average molar mass based on the added monomer-to-initiator ratio and the monomer conversion (33).

Other Cationic Polymers

Chitosan (low molar mass, degree of deacetylation 90%, batch no. BN460) was obtained from Primex (Avaldsnes, Norway). *N*-Trimethyl chitosan chloride (TMC) was synthesized by methylation of chitosan using CH_3I in the presence of a strong base (NaOH) (37). TMC25 and TMC66 were used in this work with a degree of quaternization of 25 and 66%, respectively. The degradable cationic polymer poly(2-dimethylaminoethylamino)phosphazene (PDEppz) was synthesized as reported before (10). PHPMA-DMAE was synthesized via a free radical polymerization as described previously (12). Poly(L-lysine hydrobromide) (PLL21k) was purchased from Sigma-Aldrich (St. Louis, MO, USA, lot no. 033k5118, M_w = 21,300 Da (multiangle light scattering), M_w/M_n = 1.4). Branched poly(ethylene imine) (PEI) was purchased from Sigma-Aldrich (Milwaukee, WI, USA, lot no. 05601DQ, M_w = 25,000 Da (light scattering), M_n =10,000 Da (SEC)).

Table II. PDEs Synthesized by RAFT Polymerization

Sample	[Monomer]/[RAFT]	Time (h)	Conversion (%)	$M_{n,th}$ (kDa)
PDE5	64	16	92	9.2
PDE6	640	16	85	85
PDE7	6400	16	81	810

$M_{n,th}$, theoretical number-average molar mass based on the added monomer-to-initiator ratio and the monomer conversion (34).

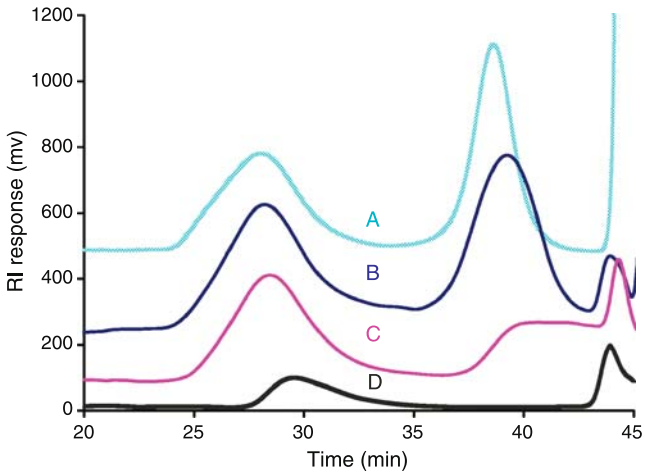

Fig. 1. Aqueous SEC chromatograms of PDE1 (low molar mass of about a few thousands) and PDE7 (high molar mass of about a few millions) in different eluents: A, 0.3 M NaAc, pH 4.4; B, 0.7 M $NaNO_3$, pH 7.2 + 10% acetonitrile; C, 0.7 M $NaNO_3$, pH 7.2; D, 0.35 M $NaNO_3$, pH 7.2.

SEC–RI System

A Waters (Milford, MA, USA) 2695 Alliance liquid chromatography system was used to perform the SEC experiments. This instrument contained a built-in autoinjector with a sample loop allowing injection of variable sample volumes and it was equipped with a Waters 2414 refractive index detector (RI). The sodium acetate eluent 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 eluent was filtrated through a 0.45- or 0.2-μm HPLC filter (Nylon, Alltech) and degassed prior to use by simultaneous application of ultrasound and vacuum.

The SEC measurements were performed with two columns, Shodex OHpak SB-806M and a precolumn Shodex SB-G in series (Showa Denko, Japan). The columns were thermostated at 30°C. The flow rate was 0.5 mL/min. The data collection and the data analysis were done with Waters Empower software.

Semipreparative Fractionation of PDEs

High molar mass PDE synthesized by RAFT polymerization was fractionated using an HPLC system containing a Waters 515 HPLC pump connected to a Waters Pump Control Module, a fraction collector (Waters II), and a differential refractometer (Waters 410). A semipreparative column set (Shodex OHpak SB-LG, OHpak SB-2006M) was used, thermostated at 30°C. The eluent was 0.3 M NaAc (pH 4.4) with a flow rate of 1.5 mL/min. For each fractionation run, 150 μL of PDE6 (Table II) with a concentration of 67 mg/mL was injected and 11 fractions were collected. Each fraction was collected during 50 s. Corresponding fractions were accumulated from 60 repeat injections. For the fractionation of PDE7 (Table II), 200 μL was injected with a concentration of 10 mg/mL and 15 fractions were collected at 40-s intervals (corresponding fractions from 120 repeat injections were pooled). The pooled fractions were dialyzed extensively against water and collected after freeze-drying.

SEC–MALS/RI System

A Waters 2690 Alliance liquid chromatography system was used to perform the SEC experiments. The analytical column series (two SB-806M columns with a precolumn) were connected online to a multiangle light scattering (MALS) detector (DAWN DSP, Wyatt Technology, Santa Barbara, CA, USA) with a He–Ne laser (λ = 633 nm) coupled to a Waters 410 differential refractometer (RI) detector (λ = 950 nm). The MALS detection measures the light-scattering intensity at 18 different angles and the RI detection measures the concentration of the polymer. This gives the absolute molar mass and root-mean-square radius of gyration for each slice. The data were processed using Astra 4.50 software. The eluent, 0.3 M NaAc (pH 4.4), filtered through a 0.02-μm film filter, was used with a flow rate of 0.5 mL/min. The column oven was set at 30°C and the RI detector at 35°C. The

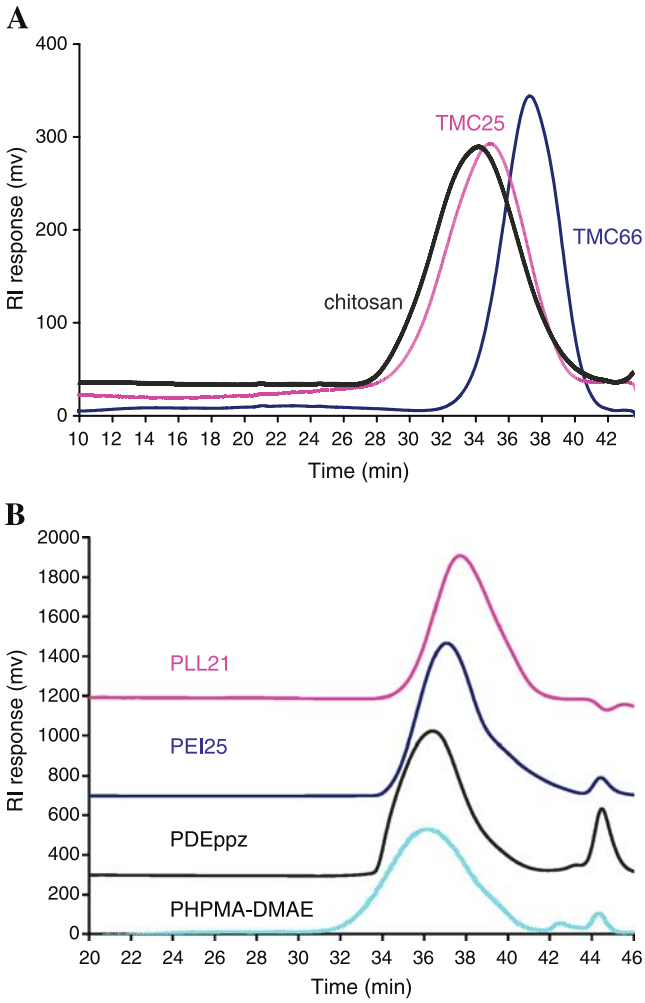

Fig. 2. SEC chromatograms of other cationic polymers used for gene delivery in eluent A (0.3 M NaAc, pH 4.4). (A) Chitosan and its derivatives TMC25 and TMC66, (B) PLL21, PEI25, PDEppz, and PHPMA-DMAE. The full names of these cationic polymers are provided in the "Materials and Methods" section.

Table III. SEC Results of Other Cationic Polymers Calibrated by PEG Standards Using Eluent A (0.3 M NaAc, pH 4.4)

Sample[a]	M_n (kDa)	M_w (kDa)	M_p (kDa)	PDI
Chitosan	39.4	177	89.4	4.49
TMC25	40.4	135	63.4	3.34
TMC66	12.4	25.7	17.8	2.07
PDEppz	18.1	32.7	28.6	1.81
PHPMA-DMAE	18.4	44.3	32.7	2.41
PEI25	8.4	20.5	19.9	2.43
PLL21	8.7	15.5	14.0	1.79

[a] The full names of these cationic polymers are provided in the "Materials and Methods" section.

refractive index increment (d*n*/d*c*) of PDE was determined by injecting polymer solutions with different concentrations (0–0.08 mg/mL) directly into the Waters 410 RI detector using a 1-mL polyethylene ether ketone (PEEK) sample loop (Rheodye, Cotati, CA, USA) with eluent at a flow rate of 0.2 mL/min. The water content of the freeze-dried polymer samples was determined by Karl Fisher titration using a Metrohm 831 KF Coulomat (Metrohm, Herisau, Switzerland). The weight concentration of the injected sample was corrected for the water content.

RESULTS AND DISCUSSION

SEC–RI

Optimization of the Aqueous SEC Conditions

In previous papers (6,13), molar masses of PDEs relative to dextran standards were determined using 0.7 M $NaNO_3$ and 0.1 M tris-(hydroxymethyl)-aminomethane (Tris) (pH 7.2) as eluent (eluent C), and two Shodex SB columns. However, the relatively high salt concentration in this eluent leads to a high viscosity of the mobile phase, which sometimes resulted in blocking of one of the columns (SB-802 column, exclusion limit about 4000, particle size 8 μm). Furthermore, when low molar mass samples were subjected to this mobile phase with the two SB-806M columns (with exclusion limit of about 20,000,000; particle size 15 μm), slightly tailing peaks were observed, which partially overlapped with the solvent peak. To optimize the eluent, PDEs with different molar masses and narrow MMDs were synthesized by living anionic and RAFT polymerization (Tables I and II), respectively. These polymers were dissolved in different eluents and injected into the same columns as mentioned above. Figure 1 shows that the low molar mass PDE1 substantially overlapped with the solvent peak when eluent C (curve C) was used. PDE4 and PDE6 (both with medium molar mass) showed slightly tailing peaks, whereas the low molar mass PDE5 displayed severe peak tailing with eluent C (data not shown). These results suggested the presence of enthalpic interactions with the stationary phase when eluent C was used, which led to errors in the determination of the molar mass and MMD. When 10% acetonitrile was added to the mobile phase (curve B in Fig. 1), less tailing was observed and better separation was obtained for low molar mass cationic polymers. This indicates that the hydrophobic interactions exist between PDE and the stationary phase that can be suppressed by adding acetonitrile to the mobile phase. When 0.35 M $NaNO_3$ and 0.05 M Tris (pH 7.2) were used, strong adsorption of PDE was observed (curve D).

NaAc (different concentrations, from 0.1 to 0.8 M) in water (adjusted to pH 4.4 with acetic acid) was also investigated as SEC eluent. Peak elution was improved (narrow peaks) and reasonable resolution was obtained, except for the eluent with 0.1 M NaAc, which showed strong adsorption and very low recovery. Representative chromatograms are also shown in Fig. 1 using 0.3 M NaAc (pH 4.4) as eluent (curve A). It can be seen from Fig. 1 that this eluent resulted in a better resolution (narrower peak) as compared to the eluent of 0.7 M $NaNO_3$ plus 10% acetonitrile (curve B).

Other eluents, such as 0.8 M $NaNO_3$, 0.02 M NaAc (pH 4.4); 0.4 M $NaNO_3$, 0.01 M NaAc (pH 4.4); and 0.8 M NaAc (pH 7.2) were also investigated. However, strong adsorption for PDE was observed for all these eluents. This indicates

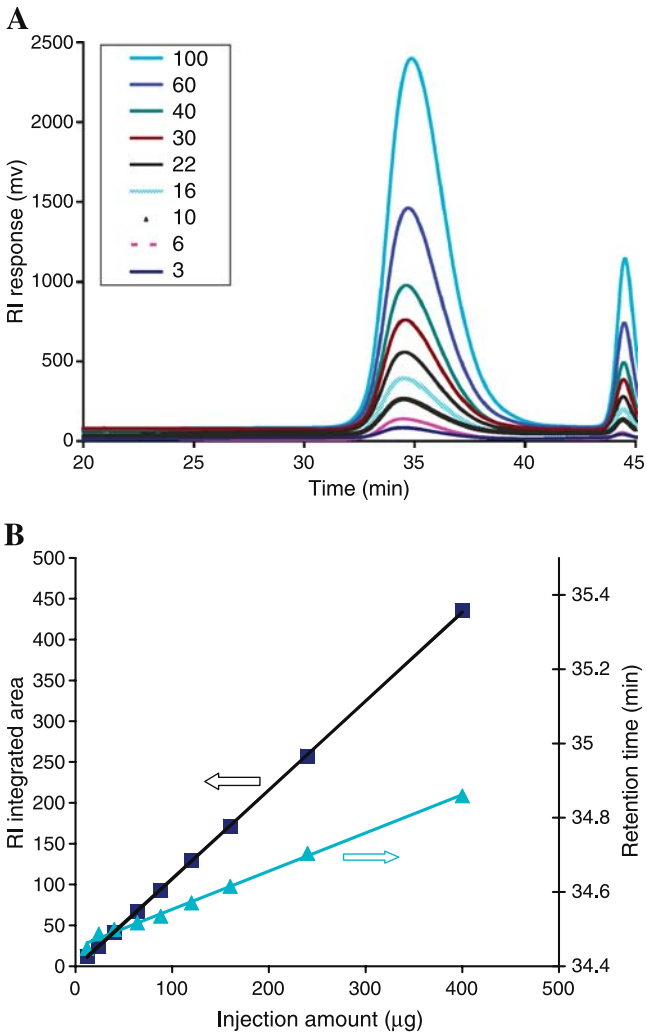

Fig. 3. Effect of the injected amount of PDE6 on (A) the SEC chromatograms, (B) the integrated area (left axis) and the retention time (right axis). Eluent A was used; PDE6 concentration was 4 mg/mL, and the variation of the injected amount was realized by varying the injection volume (from 3 to 100 μL).

Table IV. Characterization of PDE6 Fractions Calibrated with PEG Standards Using Eluent A (0.3 M NaAc, pH 4.4)

Fraction name (no.)	Retention time (min)	M_n (kDa)	M_w (kDa)	M_p (kDa)	PDI	Percentage (%)
PDE6	34.58	39.4	64.6	73.2	1.64	/
PDE6-0	33.65	103.6	124.7	115.7	1.20	1.8
PDE6-1	34.02	86.5	99.9	96.4	1.15	5.4
PDE6-2	34.29	71.4	84.7	84.4	1.19	9.9
PDE6-3	34.55	58.6	73.6	74.4	1.26	12.7
PDE6-4	34.77	47.9	65.3	66.4	1.36	14.6
PDE6-5	34.96	42.1	59.8	60.2	1.42	14.1
PDE6-6	35.12	36.0	55.1	55.7	1.53	12.7
PDE6-7	35.19	32.0	52.2	53.6	1.63	10.6
PDE6-8	35.29	28.9	49.8	51.0	1.72	8.1
PDE6-9	35.30	27.6	49.3	50.9	1.79	5.8
PDE6-10	35.34	26.4	48.4	49.7	1.83	4.3

that the good results obtained with eluent A (0.3 M NaAc, pH 4.4) are due to its (fairly high) ionic strength (0.3 M) and low pH. It should be mentioned that the pH of eluent A was adjusted to 4.4 using HAc and in this eluent about 3% (v/v) of HAc was present, which might act as organic modifier, reducing possible hydrophobic interactions between PDE and the column material.

SEC of Other Water-Soluble, Cationic Polymers

The identified conditions (eluent, column, flow rate) were used to investigate the SEC behavior of other water-soluble cationic "gene delivery" polymers. Figure 2A and B shows the SEC chromatograms of chitosan and its derivatives (TMCs), the degradable polymers polyphosphazene (PDEppz) and PHPMA-DMAE, and the "standard" polymers used in nonviral gene delivery systems, PEI25 and PLL21. The elution time shift observed in Fig. 2A for TMC25 and TMC60 indicates that some chain scission had occurred during the synthesis of these polymers starting from chitosan (38). It can be seen from Fig. 2 that the optimal SEC eluent for PDEs is also suitable for analyzing a wide range of cationic polymers used for gene delivery. The molar masses and polydispersity indices (PDIs) calibrated by PEG standards are summarized in Table III.

Effect of the Amount of Polymer Injected on SEC Elution Behavior

Figure 3A and B shows the effect of the injection volume (fixed polymer concentration) on the SEC chromatogram, integrated area, and retention time of PDE6 using eluent A. The integrated areas were proportional to the injected volume (and thus amount; Fig. 3B) in the investigated range (0.012–0.4 mg), which indicates the absence of strong enthalpic interaction (adsorption) between PDE and the stationary phase. Figure 3B also shows that the retention time slightly increased with the injection volume. At a constant injection volume (50 μL), the retention time slightly increased with increasing polymer concentration (data not shown). It has been found that with increasing polymer concentration (amount), the accompanying viscosity increases and the molecular coil size decreases, which will result in a decrease in hydrodynamic volume of the dissolved polymer (39,40). This in turn will result in an increase in SEC retention time. When the injection amount varied from 0.005 to 0.05 mg at a fixed injection volume (50 μL), the variation of the retention time of PDE6 was negligible. A similar effect of the injected amount on the SEC elution behavior (area and retention *vs.* amount) was also observed for other cationic polymers (such as PLL21 and TMC66).

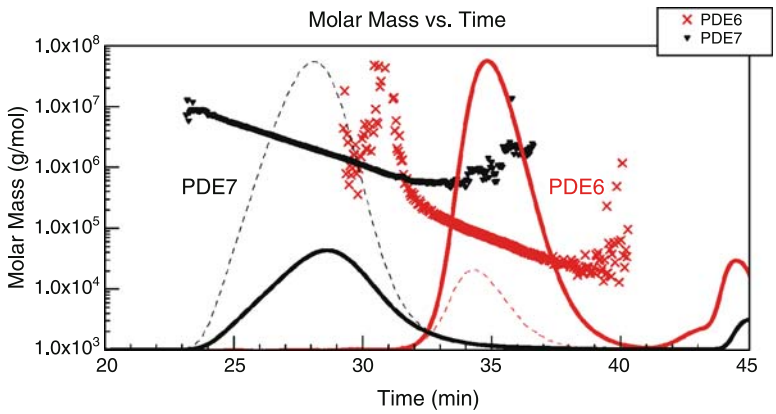

Fig. 4. The calculated dependencies of molar mass *vs.* elution time for PDE6 (peak around 34.5 min) and PDE7 (peak around 28.5 min) in eluent A. The corresponding RI (solid curves) and MALS (at 90° angle, dotted curves) chromatograms are presented.

Fractionation of High Molar Mass PDE by Preparative SEC

PDE samples with a relatively high molar mass and a low PDI are not available, and the synthesis of such polymers by state-of-the-art controlled polymerization techniques is still very difficult, if not impossible. Therefore, semipreparative aqueous SEC was applied to fractionate the high molar mass PDE6 and PDE7 using a preparative column (Shodex OHpak SB-2006M) and eluent A. The SEC results of PDE6 and the obtained fractions are summarized in Table IV. This table shows that the earlier fractions of PDE6 had high average molar masses and low PDI values. However, the later fractions showed only slight differences in molar mass and had high PDI values (some fractions, such as PDE6-8, PDE6-9, and PDE6-10, show even higher PDI values than the PDI of the feed polymer PDE6). A similar trend was observed for high molar mass polymer PDE7 (data not shown). The poorer quality of low molar mass fractions was probably due to overloading of the column. However, PDE fractions with relatively high molar mass and narrow MMD were obtained, which can be used as calibration standards for SEC (see discussion in "Calibration Curves" section).

SEC–MALS/RI

Online coupling of a molar mass-sensitive detector, such as a MALS detection, was performed to determine the absolute molar mass and MMD for cationic polymers and to verify the absence of secondary effects at the optimal aqueous SEC conditions. It should be noted that the molar mass obtained from MALS depends somewhat on the method of data processing, especially for high molar mass polymers. Based on the instructions provided by the manufacturer (Wyatt Technology) and the recommendation of Andersson *et al.* (41), zero-order Zimm extrapolation was used for low molar mass polymers (PDE1-4), first-order Zimm extrapolation for intermediate polymers (PDE6 and its fractions), and second-order Berry extrapolation for high molar mass polymers (PDE7 and its fractions). The refractive index increment d*n*/d*c* value of PDE determined in eluent A was 0.202 ± 0.006 mL/mg (three independent experiments).

Figure 4 shows the SEC–MALS chromatograms of two polymers with different molar masses (PDE6 and PDE7) and the corresponding absolute molar mass *vs.* elution time (i.e., local calibration line). It can be seen from this figure that the low molar mass PDE6 displayed a lower LS response and a higher RI response, whereas the high molar mass PDE7 showed the reverse. This is in line with expectations because the LS detector is more sensitive to polymers with high molar masses. The local calibration lines for samples PDE6 and PDE7 were straight except at both ends, where small errors in the baseline selection for both the RI and MALS signals introduced uncertainties. These two local calibration lines reasonably fit to one line over a wide molar mass range, indicating that a good SEC separation of PDE with little band broadening is obtained with the selected eluent A (0.3 M NaAc, pH 4.4) in combination with the Shodex SB-806M columns. The average values of molar mass and the MMD can be obtained from the combined measurements of molar mass (weight average, M_w) and the concentration obtained with the RI detector for each elution slice. The average values of molar mass, the PDI, the peak molar mass (M_p), and the corresponding standard deviations of samples PDE1-4, PDE6, and PDE7 are listed in Table V. Because of band broadening and that the LS detector is more sensitive for high molar mass molecules, number-average values are somewhat overestimated (but still close to the theoretical values for PDE1-4, Table I), and the resulting PDI is slightly underestimated in online SEC–MALS measurements (42).

Table V. Characterization of PDEs by Online SEC–MALS/RI (Eluent A)

Sample name	M_n (kDa)	M_w (kDa)	M_p (kDa)	PDI
PDE1	14.9 ± 0.6	17.4 ± 0.9	13.2 ± 0.3	1.16 ± 0.08
PDE2	19.3 ± 0.5	23.0 ± 0.7	16.9 ± 0.2	1.19 ± 0.05
PDE3	23.3 ± 0.5	26.4 ± 0.7	21.0 ± 0.1	1.13 ± 0.04
PDE4	29.3 ± 0.5	34.8 ± 0.7	25.5 ± 0.2	1.19 ± 0.03
PDE6	53.8 ± 3.3	69.7 ± 2.3	72.7 ± 0.8	1.30 ± 0.09
PDE7	1420 ± 40	2080 ± 40	1680 ± 30	1.46 ± 0.06

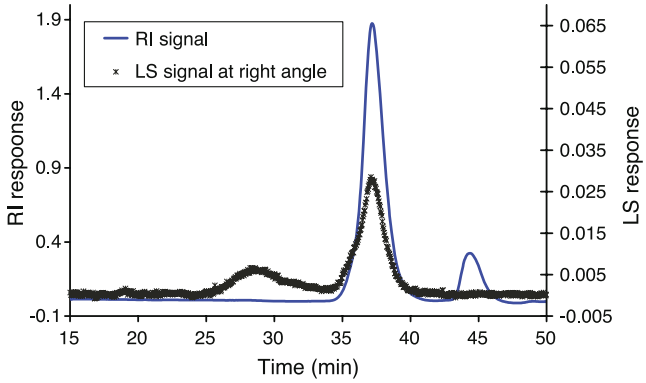

Fig. 5. SEC–MALS chromatograms (solid line, RI response; star points, LS response at 90° angle) for low molar mass PDE3 synthesized by anionic polymerization.

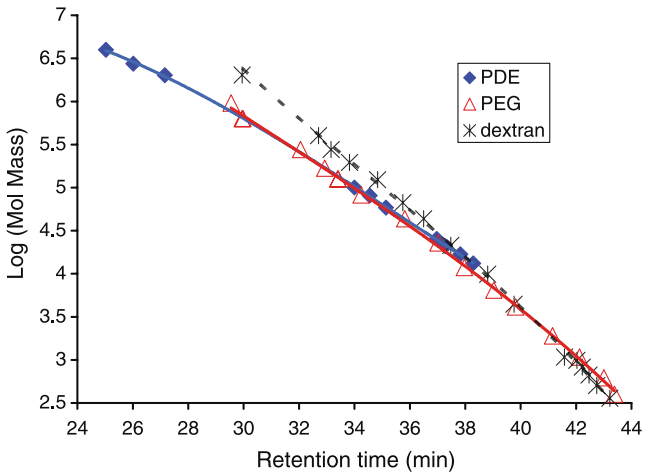

Fig. 6. Calibration curves based on different polymer standards. Diamond, PDE series; open triangle, PEG series; star, dextran series (the peak molar masses were used to construct the calibration curves).

Table VI. Characterization of PDE Fractions by Online SEC–MALS/RI (Eluent A)

Sample name	M_n (kDa)	M_w (kDa)	M_p (kDa)	PDI	R_z (nm)	R_p (nm)
PDE6-1	100.8 ± 6.0	107.5 ± 7.1	100.2 ± 2.5	1.07 ± 0.09	/	/
PDE6-3	75.1 ± 3.1	81.9 ± 3.4	80.9 ± 1.8	1.09 ± 0.06	/	/
PDE6-6	50.9 ± 3.9	64.4 ± 4.7	58.5 ± 1.7	1.27 ± 0.13	/	/
PDE7-1	3770 ± 80	4540 ± 120	3980 ± 70	1.20 ± 0.04	170.3 ± 2.6	148.2 ± 1.5
PDE7-3	2850 ± 50	3510 ± 70	2740 ± 40	1.23 ± 0.03	149.6 ± 1.7	121.7 ± 1.4
PDE7-5	2070 ± 40	2660 ± 50	2020 ± 20	1.28 ± 0.03	131.1 ± 1.9	101.7 ± 1.3

R_z, z average root-mean square radius of gyration; R_p, root-mean-square radius of gyration at the elution peak maximum. Because the R_z and R_p for PDE6 and its fractions are too small to obtain an accurate value, they are not included in this table.

An example of the SEC–MALS chromatograms of a low molar mass PDE synthesized using anionic polymerization is shown in Fig. 5. The LS chromatogram of PDE3 showed two separate peaks, whereas its RI chromatogram only showed one peak (the peak at 45 min is due to the injection of solvent). Similar SEC–MALS elution profiles were observed for other PDE polymers (PDE1, PDE2, and PDE4) synthesized by anionic polymerization, but not for the polymers obtained by RAFT polymerization. The earlier-eluting peak with a high molar mass represents less than 1% of the sample based on RI response. This fraction is neither due to contamination from previous injections ("carryover") nor to reversible aggregation. (We collected two effluent fractions for PDE3 sample corresponding to the two peaks, and then reinjected them on the original SEC system. Only one individual peak was observed for each fraction at the same elution position as found in the unfractionated sample.) It is an unknown inherent contaminant, which is probably related to the anionic polymerization process. For example, Wyatt *et al.* (43) reported a dimer contaminant in the SEC–MALS chromatogram of narrow polystyrene standards, which were prepared, in general, by anionic polymerization. Whatever the reason for this high molar mass fraction, it is demonstrated that MALS is a very powerful technique for detecting the presence of such a fraction, which will not be noticed using convential RI detection.

The absolute molar mass information and the root-mean-square radius (rmsR) of gyration for the fractions of PDE6 and PDE7 were also determined by SEC–MALS (Table VI).

Calibration Curves

Figure 6 shows the calibration curve in semilogarithmic coordinates obtained using low molar mass PDE1-4 and some fractions of PDE6 and PDE7 (the peak molar masses in Tables V and VI were used). For comparison, calibration curves obtained from commercial standards (PEG series and dextran series) are also shown. This broad-range calibration curve (obtained by injecting several reference standards with narrow MMD across a broad range of molar masses) is independent of band broadening because only peak molar masses are used. In contrast, the local calibration curve obtained from one sample using online SEC–MALS as described above is affected by SEC band broadening (44). It can be seen from Fig. 6 that the PDE calibration curve coincided with the PEG curve (especially in the molar mass range of 25,000–300,000), whereas it deviated from that of dextran, especially in the high molar mass range (above 10,000 Da). Thus, the apparent molar mass averages (M_n and M_w) of PDE polymer obtained using PEG standards resulted in smaller errors than those obtained using dextran standards, as can be seen in Table VII. For the high molar mass PDE polymers, such as PDE7, not only the relative values of molar mass averages (based on dextran standards), but also the corresponding PDIs were largely overestimated. This is because the hydrodynamic volume (size) of the dextran standards, which features some branches, is smaller than that of a linear polymer such as PEG. This difference increases with increasing molar mass, because high molar mass dextrans include more branched structures. It can be seen from Tables I and VII that the M_n values of PDE1, PDE2, and PDE4 calibrated by PDEs are very close to the M_n values obtained by NMR measurements. Therefore, narrow-distribution PDEs should be used for calibration to obtain reliable molar mass and MMD of PDE samples. If this is difficult, it is better to use linear polymer standards, such as PEG, than branched dextran standards. For other cationic polymers, similar procedures as described in this paper can be used to obtain their absolute molar masses and MMDs.

Table VII. Comparison of the Molar Masses and MMDs of Different PDEs Using Different Calibration Standards (Eluent A)

	Dextran, equivalent value				PEG, equivalent value				PDE, equivalent value			
Sample name	M_n (kDa)	M_w kDa	M_p (kDa)	PDI	M_n (kDa)	M_w kDa	M_p (kDa)	PDI	M_n (kDa)	M_w kDa	M_p (kDa)	PDI
PDE1	10.0	17.7	15.6	1.77	8.2	12.9	11.7	1.58	11.2	14.2	13.4	1.27
PDE2	17.1	25.3	21.3	1.48	12.9	17.6	15.5	1.36	15.3	18.6	16.7	1.22
PDE3	24.3	33.4	29.7	1.37	17.5	22.7	20.8	1.30	19.4	23.0	21.4	1.19
PDE4	31.0	43.8	35.9	1.41	22.0	29.0	24.6	1.32	23.2	28.4	24.6	1.22
PDE5	10.7	16.7	18.1	1.56	8.4	12.2	13.4	1.45	11.2	13.8	15.0	1.24
PDE6	61.1	118	131	1.93	39.4	64.6	73.2	1.64	41.9	62.2	69.6	1.49
PDE7	3540	15670	8150	4.43	1080	2150	1720	2.00	1010	1810	1600	1.79

CONCLUSIONS

Optimal aqueous SEC conditions for cationic PDEs with a broad molar mass range were obtained: the combination of eluent A (0.3 M NaAc, pH 4.4) with the Shodex SB-806M column series. These conditions were also shown to be suitable for other cationic polymers used in gene delivery, such as chitosan and its derivatives, PEI, PLL, polyphosphazene, and PHPMA-DMAE.

The absolute molar masses and distributions of different PDEs were obtained directly from online SEC–MALS/RI. The results demonstrate that a good SEC separation of PDEs with little band broadening was achieved. Using narrow PDEs, a reliable broad-range calibration curve was established for routine SEC analysis of PDEs. Because standards of known molar masses with narrow distributions are not commercially available for most polymers used in pharmaceutics and biotechnology, the procedure described in this work can also be applied for molar mass characterization of other water-soluble polymers.

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