Characterization of Poly[di(carboxylatophenoxy)-phosphazene] by an Aqueous Gel Permeation Chromatography

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SYNOPSIS

A water-soluble phosphazene polyelectrolyte, poly[di(carboxylatophenoxy)phosphazene] (PCPP), was characterized using aqueous gel-permeation chromatography (GPC) with concentration (UV and RI) and molecular weight sensitive (multiangle laser light-scattering) detectors. Agreement was observed between the weight-average molecular weights determined by GPC with a light-scattering detector, conventional GPC using fractionated narrow PCPP standards, and also by static light-scattering measurements. The effect of chromatography conditions, such as ionic strength of the mobile phase, column resolution, and injection volume was investigated. Mark–Houwink constants of PCPP in aqueous solution (phosphate buffer, pH 7.4, 0.42*M* NaCl) were determined. The validity of the universal calibration curve and the occurrence of a secondary nonexclusion mechanism of separation in aqueous GPC of phosphazene polyelectrolytes are discussed. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

The characterization of polyorganophosphazenes by gel permeation chromatography (GPC) can only be achieved with great care. Anomalous behavior of phosphazene polymers was reported for different eluent-stationary phase pairs and resulted in distorted, severely tailing, and nonreproducible chromatograms.[1,2] These anomalies were explained by the adsorption type of interaction between the polymer and the column materials and by formation of aggregates due to the presence of residual side groups. Different solutions to the problem were suggested, mostly based on the addition of ionic species, such as tetrabutylammonium bromide, LiBr, or KCl to the organic solvents, which in most cases led to the successful characterization of the polymers.[1,2] However, little or no information exists regarding GPC analysis of polymers with a phosphazene backbone in an aqueous medium. Characterization of water-soluble phosphazene polyelectrolytes can be especially challenging due to the presence of charged groups on the macromolecular chain, which, as is known for other polyelectrolytes, may affect the separation via secondary nonexclusion mechanisms.[3] Meanwhile, water-soluble phosphazene polyelectrolytes, such as poly[di(carboxylatophenoxy)phosphazene] (PCPP), are of considerable interest because of their unique physicochemical and biological properties and their important applications as materials for microencapsulation and immunoadjuvants.[4-7] Aqueous GPC characterization of PCPP becomes of major importance because of polymer insolubility in common organic solvents.

Recently, an attempt was made to use aqueous GPC in the degradation study of ionotropic polyphosphazene gel microspheres.[8] Changes in GPC peaks of the phosphazene polymers were monitored and the relative magnitudes of molecular weights were estimated in polyacrylic units. However, a systematic approach to the GPC analysis of phosphazene polyelectrolytes was not developed and their absolute molecular weights were not determined. In the present article, we have tried to develop and optimize a method for the determination of absolute molecular weight for the phosphazene polyelectrolyte, PCPP, utilizing aqueous GPC with concentration (UV and RI) and molecular weight-sensitive,

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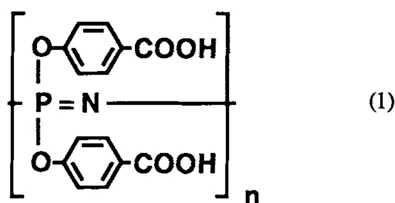

(1)

Scheme 1

multiangle laser light-scattering (MALLS) detectors. Our objective was also to investigate the influence of experimental parameters in order to evaluate and minimize nonexclusion effects and to get the most accurate characterization of phosphazene polyelectrolyte for both narrow and broad molecular weight distributions.

EXPERIMENTAL SECTION

The chromatographic system was equipped with a Shodex DEGAS KT-37 on-line vacuum degasser (Showa Denko K.K., Tokyo, Japan), a Waters 510 HPLC pump with pulse dampener (Waters, Milford, MA), two in-line filters—0.5 micron high-pressure filter (Rainin, Woburn, MA) and 0.02 micron filter (Anodisc 25, Whatman International Limited, Maidstone, England) in a High Pressure Stainless Filter Holder (Millipore, Bedford, MA) and a Waters 717plus Autosampler. Two different column sets were used. The first set (UH linear) consisted of an Ultrahydrogel guard column and an Ultrahydrogel Linear column, which is a mixture of packing of different porosities (Waters, Milford, MA). The second set (UH 2000 + 250 + 120) included the Ultrahydrogel guard column, Ultrahydrogel 2000 (pore size 2000 Å), Ultrahydrogel 250 (pore size 250 Å), and Ultrahydrogel 120 (pore size 120 Å) columns connected in a series. The column size for all columns was 7.8 mm i.d. × 30 cm. Both of the column sets were maintained at 35°C in a Waters column oven. The HPLC system also included a Waters 486 tunable UV/visible absorbance detector set at 254 nm connected in series to a multiangle laser light-scattering (MALLS) detector (DAWN DSP-F, Wyatt Technology, Santa Barbara, CA), and a Waters 410 refractive index detector. The refractive index detector was calibrated using KCl as the standard.[9]

Phosphate buffer solution (PBS) (pH 7.4)—Dulbecco's phosphate-buffered saline (Sigma Chemical Co., St. Louis, MO)—was used as a mobile phase. While this buffer contains Na_2HPO_4, KH_2PO_4, NaCl, and KCl as components, its ionic strength is mainly determined by the concentration of NaCl. PBS, with concentration of NaCl 0.42*M* was used unless otherwise noted. Mobile phases were filtered through a 0.02 micron filter (Anodisc 47, Whatman International Limited, Maidstone, England) into a four-valve Ultra-Ware filtration reservoir (Kontes, Vineland, NJ). NaN_3 (0.01%) was added to mobile phase to prevent biological degradation of the columns.

Astra 2.1 data capture and processing software (Wyatt Technology, Santa Barbara, CA) was used to calculate molecular weight averages based on MALLS detection data. The refractive index increment (dn/dc) of PCPP was determined by injecting known concentrations of PCPP and using Astra 2.1 software assuming 100% mass recovery and known RI calibration constant. The results were then averaged. Millenium (Waters, Milford, MA) software was used to obtain molecular weight distributions from conventional GPC with UV and RI detection.

Broad molecular weight samples of PCPP were synthesized as described elsewhere.[4] Poly(ethylene oxide) (PEO) standards (Tosoh Corporation, Tokyo, Japan) were used as received. Narrow PCPP standards were obtained by chromatographic fractionation of broad PCPP using a Pharmacia HPLC system including a P-500 pump, LCC-500 plus controller, UV-1 detector, and Frac-100 fraction collector (Pharmacia Biotech, Uppsala, Sweden). A Phamacia XK 50/60 column packed with Sepharose CL-6B (Pharmacia, Uppsala, Sweden) was used, with 0.1*M* ammonium bicarbonate buffer (pH 8.0) as a mobile phase. The fractionated PCPP was then lyophilized on a Labconco freeze dryer 8 (Labconco Kansas City, MO). PCPP samples were prepared for GPC using equivalents of 0.1*M* NaOH and incubated at 55°C until dissolved in a New Brunswick G24 Environmenal Incubator Shaker (New Brunswick Scientific, Edison, NJ). The samples were then

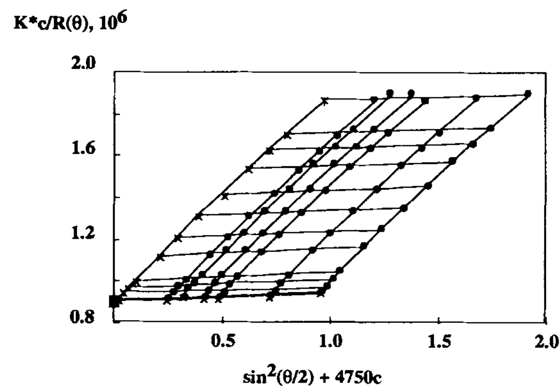

Figure 1 Zimm plot for broad PCPP sample (PBS, pH 7.4, 37°C).

Table I Characterization of Broad PCPP Sample Using Static MALLS (pH 7.4, 37°C)

	dn/dc (mL/g)	A_2 (mol mL/g^2)	$M_w \times 10^{-3}$ (g/mol)	R_z (nm)
PCPP-BS	0.272	$1.1 \cdot 10^{-4}$	1,110	73

diluted to a 0.1% w/v solution in a mobile phase for GPC injection. PEO standards were dissolved in a mobile phase. The samples were filtered with Millex HV 0.45 micron filter units (Millipore, Bedford, MA).

Viscosities of polymer samples were measured at 35°C using an Ubbelohde viscometer (Ertco Precision, West Patterson, NJ) with the buffers employed as mobile phases for the GPC experiments. Intrinsic viscosities [η] were determined by the extrapolation of specific viscosities to zero concentration according to Huggins equation.[10]

RESULTS AND DISCUSSION

Characterization of Broad and Narrow PCPP Samples

PCPP (Scheme 1) was synthesized by macromolecular substitution of poly[di(chloro)phosphazene].[4] Narrow PCPP standards were obtained by fractionation of broad PCPP samples using GPC with ammonium bicarbonate as a mobile phase with subsequent lyophylization of the collected fractions.

A PCPP sample was characterized in a phosphate buffer containing 0.42*M* NaCl at pH 7.4 using static MALLS technique to evaluate the weight-average molecular weight (M_w), second virial coefficient (A_2), and *z*-average root mean square radius (R_z) (Table I). The Zimm plot (Fig. 1) shows no anomalies that might suggest the presence of microgels and reveals relatively low values for A_2, suggesting that PBS with 0.42*M* NaCl is a poor solvent for PCPP. Slow precipitation is observed at 35°C as the concentration of NaCl in the buffer is raised from 0.42*M* to 0.56*M*, indicating near theta conditions.

Figure 2 shows an overlay of GPC chromatograms of a broad PCPP sample obtained using different detectors—(1) MALLS, (2) UV, and (3) RI. As seen from the figure, the light-scattering peak is displaced toward the high molecular weight end of chromatogram compared to the concentration detection peaks. Because the detectors were already aligned for interdetector volume differences, the displacement of the GPC-MALLS peak is caused entirely by the lower sensitivity of the light-scattering detector over the low-molecular weight section of the chromatogram associated with low concentration and small Rayleigh ratios of late eluting fractions of the sample. At the same time GPC-MALLS and UV-GPC chromatograms of narrow PCPP sample are practically superimposable even in the low-molecular weight range (Fig. 3, curves 1 and 2, correspondingly), which demonstrates that the GPC-MALLS method is a powerful tool for their characterization. The molecular weight parameters of these narrow samples were determined by GPC-

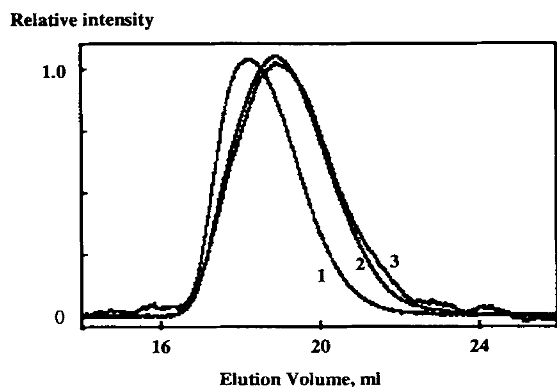

Figure 2 Chromatograms of broad PCPP sample obtained using MALLS (1), UV (2) and RI (3) detection (PBS, pH 7.4, 37°C, column set—UH 2000 + 250 + 120).

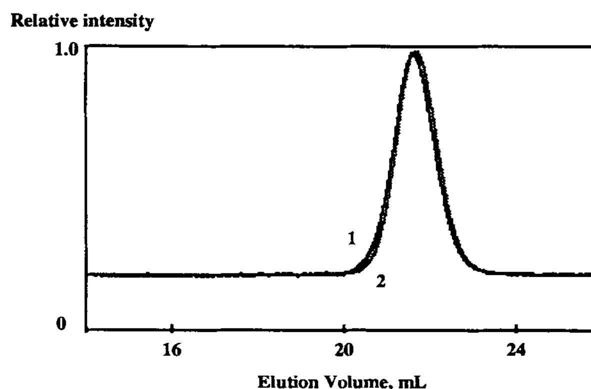

Figure 3 Chromatograms of narrow PCPP standard obtained using MALLS (1) and UV (2) detection (PBS, pH 7.4, 37°C, column set—UH 2000 + 250 + 120).

Table II Molecular Weight Averages of PCPP Narrow Standards Obtained by GPC-MALLS (pH 7.4; 37°C; Column Set: UH 2000 + 250 + 120)

Sample	$M_w \times 10^{-3}$ (g/mol)	$M_n \times 10^{-3}$ (g/mol)	M_w/M_n
NS-1	2.7	2.6	1.0
NS-2	4.4	3.8	1.2
NS-3	32	29	1.1
NS-4	37	35	1.1
NS-5	38	34	1.1
NS-6	62	58	1.1
NS-7	80	73	1.1
NS-8	420	330	1.3
NS-9	470	360	1.3
NS-10	590	480	1.3
NS-11	690	540	1.3
NS-12	770	640	1.2
NS-13	2500	1660	1.5

MALLS (Table II) and used to build the conventional calibration curves for the UV and RI detectors. Applicability of the MALLS technique to the characterization of macromolecules of extremely low molecular weights, if they are present at sufficient concentration, was demonstrated earlier.[11]

Table III presents molecular weight averages and polydispersity parameters determined for the broad PCPP sample. Good agreement between M_w obtained by GPC-MALLS and static MALLS was demonstrated. Correlation between GPC-MALLS and conventional GPC is excellent for M_w values, but much less so for M_n, which is consistent with the differences in chromatograms discussed above. Because the sensitivity of the detector at the low molecular weight end of the peak has significant impact on the determination of the number-average molecular weight, the "unobserved" portion of GPC-MALLS chromatogram can be an explanation for the higher, overestimated number-average molecular weight. Examination of the MALLS molecular weights as a function of elution volume proves that the reliable regime for broad PCPP chromatogram does not include the low molecular section because of the high baseline noise (Fig. 4, crosses). The conventional calibration curve (molecular weights vs. elution volume) obtained using narrow standards characterized by GPC-MALLS (Fig. 4, dashed line, empty spheres—datapoints) overlays with the broad sample curve in most of the peak area (RI signal is superimposed in the figure), proving its applicability for the analysis and significantly lower error in the low-molecular weight area. However, Figure 4 also reveals minor deviations in linearity in the molecular weight vs. elution volume for the broad sample that can be attributed to branching variances over the molecular weight range of the sample,[12] which apparently may not be accounted for by using conventional calibration in the case of PCPP.

The differences in the M_n obtained using UV and RI detectors can also be explained by the fact that

Table III Comparison of Different Detection Methods in the GPC Analysis (Number of Replicates: 7; pH 7.4; 37°C; Column Set: UH 2000 + 250 + 120)

	MALLS	GPC-MALLS	GPC-RI	GPC-UV
$M_w \times 10^{-3}$, g/mol	1,110	1,220	1,220	1,190
Standard deviation, %		8	8	6
$M_n \times 10^{-3}$, g/mol		690	520	450
Standard deviation, %		10	21	4
M_w/M_n		1.8	2.3	2.6
Peak elution volume, mL			27.49	27.10
Standard deviation, %			0.8	0.4

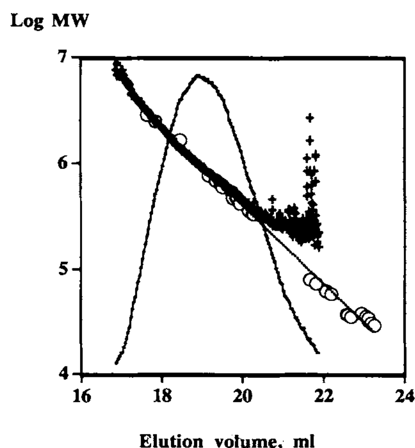

Figure 4 Log molecular weight vs. elution volume for broad PCPP sample (crosses) and for narrow PCPP standards—conventional calibration curve (dashed line, empty spheres—datapoints). The RI signal is superimposed (PBS, pH 7.4, 37°C, column set—UH 2000 + 250 + 120).

M_n values generally depend to a great extent on baseline noise.[13-15] Thus, the higher signal-to-noise ratio observed for the UV detection of PCPP leads to lower values and significantly smaller deviations in determination of number-average molecular weight compared to the RI detection method (Table III, Fig. 2).

Effect of GPC Parameters on the MW Determination

Ionic strength of the mobile phase is an important parameter in the chromatographic separation of polyelectrolytes.[16] An adequate concentration of low molecular weight salt is usually highly desirable to

Table IV Molecular Weight Distributions of Broad PCPP Samples Obtained Using Linear Column Set (MALLS, UV Detection, pH 7.4, 37°C)

	GPC-MALLS	GPC-UV
$M_w \times 10^{-3}$, g/mol	1,230	1,200
$M_n \times 10^{-3}$, g/mol	780	370
M_w/M_n	1.6	3.2

suppress secondary nonexclusion effects and achieve high resolution during the analysis. Change in the concentration of NaCl in the mobile phase from 0.42M (highest possible before PCPP starts to precipitate) to 0.14 M did not affect the values of molecular weight averages including M_n obtained by GPC-MALLS. The effect of the injection volume (15–75 μL) on the molecular weight parameters of PCPP determined by GPC-MALLS and conventional calibration GPC was also evaluated and revealed good correlation in the results.

The effect of the column resolution on the molecular weight analysis of PCPP was studied using a bank of three columns containing packing of pore sizes 2000 Å, 250 Å, and 120 Å, and a linear column comprising mixed packing with the same pore size range 2000–120Å. The linear column also had a longer application history in the analysis of water-soluble polymers of different types. Figure 5 shows that both column sets separated narrow PCPP standards well, although the calibration curve for the column bank is flatter. Thus, the important parameter determining GPC resolution and accuracy—

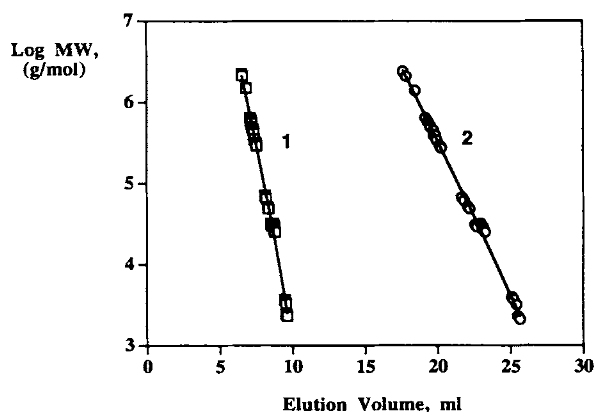

Figure 5 Conventional calibration curve for UH linear (1) and UH 2000 + 250 + 120 (2) column sets obtained using PCPP narrow standards (UV detection, PBS, pH 7.4, 37°C).

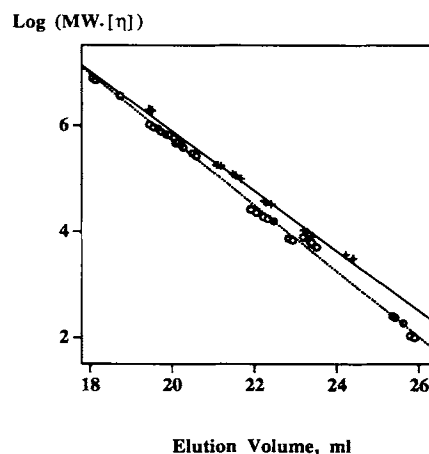

Figure 6 Universal calibration curves based on PEO standards (solid line, crosses—datapoints) and narrow PCPP standards (dashed line, empty spheres—datapoints) (PBS, pH 7.4, 37°C, column set—UH 2000 + 250 + 120).

GPC selectivity, defined as the ratio of the difference in elution volume to the difference in the logarithm of the molecular weight (the inverse slope of the calibration curve)[17]—is lower for the linear column and, thus better molecular weight resolution can be expected using the column bank. No differences were observed for the values of M_w obtained on these columns using both MALLS detection and conventional calibration with UV detection (Tables III and IV). However, the value for the M_n obtained for linear column using MALLS detection is slightly higher (about 15%). M_n determined by conventional GPC using linear column is, on the contrary, lower. This apparent contradiction can be probably explained by the poorer resolution and even existence of some nonspecific interactions for the linear column modified during its long application history. The effects should obviously cause the decrease of M_n as determined by the conventional GPC. However, nonmonodispersity of each elution volume increment resulting from the poor resolution can lead to the overestimation of M_n determined by MALLS, because this method is more sensitive to the high molecular weight fractions.

Universal Calibration

In the molecular weight determination of uncharged polymers, the validity of a universal calibration curve for different types of polymers has been widely demonstrated. The key parameter in this method is the so-called universal calibration function ($M \times [\eta]$) value proportional to the hydrodynamic volume of the molecule. If size exclusion is the only mechanism that controls the separation, a plot of $\log(M \times [\eta])$ vs. elution volume should yield the same universal curve for a given chromatographic column irrespective of the chemical structure of the polymer. The calibration curve can be constructed using polymer standards with known molecular weights and Mark–Houwink constants—parameters in the equation linking intrinsic viscosity and the molecular weight of the polymer ($[\eta] = KM^{\alpha}$). Therefore, the intrinsic viscosities of PCPP and PEO (relative standard for the universal calibration) samples were determined at 35°C using the GPC mobile phase as a solvent. Mark–Houwink constants were then calculated based on the molecular weight data obtained for these samples by GPC-MALLS. The values of K and α were 2.93×10^{-4} dL/g and 0.622, respectively for PCPP, and 2.65×10^{-3} dL/g and 0.536 for PEO.

The molecular weight averages obtained for the broad PCPP sample were compared using universal calibration of the column based on uncharged PEO standards and GPC-MALLS data. It was found that universal calibration leads to the overestimated values of molecular weights ($M_w = 1{,}490 \cdot 10^3$, $M_n = 730 \cdot 10^3$ g/mol using universal calibration vs. $M_w = 1{,}220 \cdot 10^3$, $M_n = 690 \cdot 10^3$ g/mol using GPC-MALLS), suggesting the presence of secondary nonexclusion mechanisms in the separation. To analyze this disagreement it is necessary to compare universal calibration graphs obtained under the same experimental conditions for both polyphosphazene polyelectrolyte and uncharged PEO. Figure 6 shows that the deviation of the elution volume toward lower values is observed for PCPP compared to the reference calibration for PEO standards. This can be apparently explained by so-called ion-exclusion effect resulting from the existence of electrostatic repulsion interactions between column material and polyelectrolyte.[3,18–20] The effect is based on the fact that many commercial packings for GPC, including Ultrahydrogel's polymethacrylate-based gel, bear some degree of negative charges[21] that prevent polyions from freely diffusing into the pores of the gel matrix. As a result, peaks elute earlier than expected for neutral polymers of the same size and the molecular weight calculated by universal calibration is overestimated. As seen from Figure 6, the deviation from the ideal behavior is especially remarkable for the low molecular weight polymers that can usually penetrate a larger pore volume, which is similar to other findings.[3] The effect can be effectively described as a reduction of the pore volume accessible for polyanions. Attempts to quantify this phenomenon were made and a so-called "repulsion length" value was defined as a difference between the geometric pore radius and the apparent (diminished) effective pore radius.[19] It is noteworthy that the repulsion interactions can be noticeable even in the relatively high ionic strength range used for the PCPP analysis, which correlates with data for some other polyelectrolytes reported earlier.[3,22] The electrostatic exclusion is apparently highly dependent on the application history of the column.[22]

CONCLUSIONS

Good agreement was demonstrated in M_w values of broad PCPP samples determined by static MALLS, GPC-MALLS, and conventional GPC using narrow polyphosphazene standards. Separation based on a size exclusion mechanism was achieved for narrow and broad PCPP samples, resulting in a reproducible determination of M_n. The accuracy of M_n measure-

ments seems to be somewhat higher for GPC with concentration detectors than for GPC with light-scattering detector. However, application of the GPC-MALLS to the analysis of PCPP samples appears to be preferable because of the higher sensitivity of conventional GPC to the secondary nonexclusion mechanisms and because the assumption has to be made in this method that the samples and the standards have the identical structural characteristics. Failure of universal calibration based on noncharged PEO standards in the PCPP analysis was demonstrated apparently due to some interference of ion-exclusion mechanisms with size exclusion separation.

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