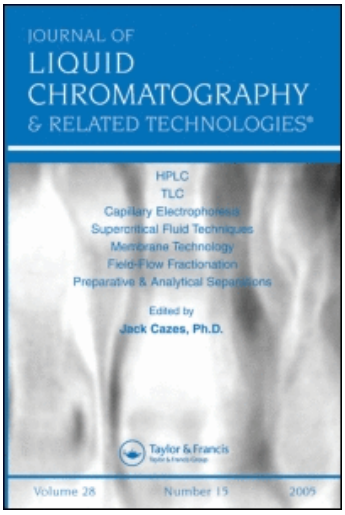

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Molar Mass Distribution of a Polymer-Drug Conjugate Containing the Antitumor Drug Paclitaxel by Size Exclusion Chromatography and Universal Calibration

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MOLAR MASS DISTRIBUTION OF A POLYMER-DRUG CONJUGATE CONTAINING THE ANTITUMOR DRUG PACLITAXEL BY SIZE EXCLUSION CHROMATOGRAPHY AND UNIVERSAL CALIBRATION

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ABSTRACT

The development of a Size Exclusion Chromatography (SEC) method for the characterization of the polymer-drug conjugate PNU 166945 is presented. PNU 166945 is a conjugate between poly[N-(2-hydroxypropyl) methacrylamide)] and the antitumor drug paclitaxel. This study follows a previous paper on a similar polymer conjugate, FCE 28068, containing the same polymeric carrier and the antitumor drug doxorubicin. The aim was an accurate and reproducible, yet relatively simple and rapid method for the routine quality control of production batches.

Thirteen PNU 166945 narrow fractions were separated, by semi-preparative SEC, and characterized by on-line SEC-Viscometry and off-line Light Scattering. The fractions allowed us to verify that PNU 166945 follows the universal calibration constructed with PEO/PEG narrow standards. So a conventional SEC method that utilizes just a refractive index detector and universal calibration with commercial narrow standards was found suitable. The study also reports data on accuracy and reproducibility of the SEC measurement.

INTRODUCTION

Interest in the area of polymeric drug delivery systems for cancer chemotherapy is increasing considerably, especially after the regulatory authorithy approval of two products, styrene maleic anhydride-neocarzinostatin (SMANCS) and polyethylenglycol-L-asparaginase.[1,2] Polymer-based drug delivery systems are usually designed to improve the pharmaco-kinetic profile of an antitumor agent, by improving tumor specific targeting, and allowing long-term controlled release. In advantage to other macromolecular carriers like immuno-conjugates or natural polymers, synthetic polymers offer the possibility of optimizing features, such as molar mass and inclusion of targeting moieties. On the other hand, synthetic polymers are inherently heterogeneous, as far as molar mass distribution (MMD) and drug loading/distribution are concerned. This poses complex problems to the analytical characterization of any investigational new drug, which must "assure a final product that meets identity, strength, and purity specifications".[3] Achievement of this goal proved already feasible for polymer-based drug delivery systems: for example, during the recent development of FCE 28068, a Poly[N-(2-hydroxypropyl)methacryl amide)] (PHPMA) copolymer bound to the antitumor drug doxorubicin, validated methods were set-up, which met the above cited requirements.[4-6] In particular, a Size Exclusion Chromatography (SEC) method,[5] that utilizes refractive index detection and relies upon universal calibration with commercial standards, was used to determine the polymer MMD. This is an important polymer feature: it can maximize the therapeutic index of the drug, by influencing the plasma clearance and the polymer accessibility to target cells other than phagocytes.[7] Besides, accumulation of non-degradable polymers in the body may occur if their molar mass is higher than the renal excretion threshold. Here, a conventional SEC method is presented for the determination of the average molar mass and its distribution, as performed on a polymer-paclitaxel conjugate, PNU 166945.

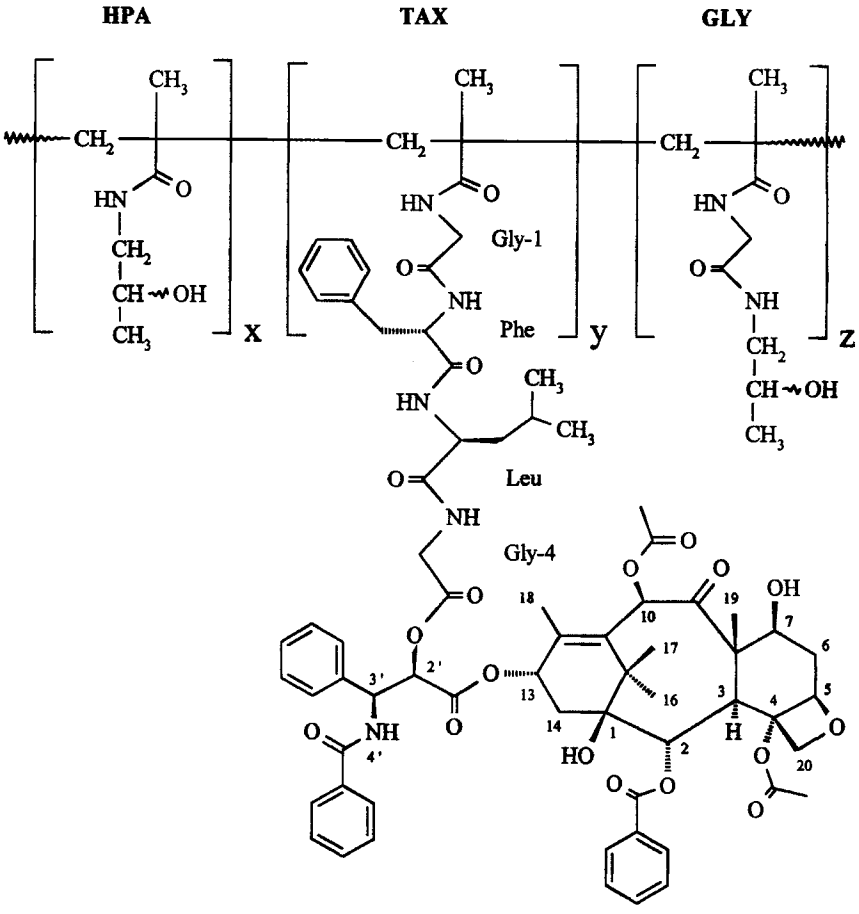

Figure 1. Structure of PNU 166945 a random copolymer based on poly-HPMA (predominant monomer) and containing paclitaxel molecules connected to the polymer backbone through a tetrapeptide spacer.

Paclitaxel (a diterpene) possesses anticancer activity in ovarian, breast, and non-small cell lung cancer, as well as other cancer types.[8] Because of its aqueous insolubility, paclitaxel is administered in ethanol and cremophor-EL. In the initial clinical studies, various observed hypersensitivity reactions were ascribed to cremophor.[8] PNU 166945, Figure 1, is a water - soluble copolymer of [methacryloyl-glycyl-phenylalanyl-leucyl-glycyl-paclitaxel], [1-methacryloylamide-2-hydroxy-propane] and [1-(methacryloylglycyl)amino-2-hydroxypropane].

Improved antitumor activity of PNU 166945, compared to free drug has been demonstrated preclinically.[9] At present, the compound is under phase I clinical evaluation.

This study follows the scheme of the above cited work on a similar conjugate polymer: FCE 28068.[5] Many of the results obtained in the previous study are here assumed. The aim of the SEC method here described was a relatively simple and fast procedure, complying with the batch quality control needs, for the accurate and reproducible determination of the true MMD and relative weight-average molar mass (Mw). The method implied: construction of universal calibration in the chosen experimental conditions, using commercial narrow standards as Poly(ethylen oxide) (PEO), and Poly(ethylen glycol) (PEG); fractionation of PNU 166945; check of universal calibration for the narrow fractions of PNU 166945; check of the SEC measurement accuracy by comparison with results of independent light scattering determinations; check of the SEC measurement reproducibility. The study utilized some classic analytical techniques for the characterization of macromolecules in solution, such as multi-angle laser light scattering (MALLS) and viscometry both off-line and on-line with the SEC system.

MATERIALS AND METHODS

Source of Polymers

Seven PEO narrow MMD standards were purchased by Shova Denko. Six PEG narrow standards were purchased from Polymer Laboratories. PNU 166945 (lot OF 11361/68) and analogs with reduced or no paclitaxel content (lots OF11361/01 and OF 11361/82 respectively) were Pharmacia & Upjohn products. PNU 166945 fractions were obtained as described below. Six PHPMA homopolymer fractions were obtained from Dr. K. Ulbrich (Institute of Macromolecular Chemistry, Czech Academy of Sciences, Prague).

PNU 166945 Fractionation

Four lots of PNU 166945, with sizeably different Mw values, have been prepared as starting material for the preparation of narrow MMD fractions, as described elsewhere.[10] Tailoring of the polymer molar mass was obtained by varying the concentration of the polymerization initiator in the synthesis of the polymeric precursor.[11] PNU 166945 lots were fractionated on a 5x100 cm column containing a 1:1 mixture of Sepharose 4B and 6B (Pharmacia Biotech). The eluent was 50 mM sodium acetate, 0.5 M NaCl, pH 5.0; flow-rate 1 mL/min; temperature

10°C; loaded sample concentration 1 g/10 mL. The polymer elution peak was monitored off-line by optical density measurement at 250 nm on a 8451A UV-visible detector (Hewlett-Packard) after proper dilution. The chromatographic peak of PNU 166945 was integrated and the integrated surface divided into 7 fractions of similar area. Tubes corresponding to each fraction were pooled and concentrated in an Amicon 8050 or 8400 ultrafiltration cell on a Diaflo YM membrane (cut-off 10000, 5000, 2000, 1000, depending on the fraction average molar mass) under nitrogen pressure (3.7 atm).

Re-concentrated samples were subjected to further chromatographic steps, both to thoroughly desalt them and to reduce polydispersity. According to the fraction average molar mass, the 2.6x25 cm column was filled with Superdex 200 or Superdex 75 (all from Pharmacia Biotech). The elution was monitored on-line by both a UV-visible detector and a conductimeter (through a micro-flow cell). Eluent: 1 mM trifluoroacetic acid, pH 3.0; flow-rate 1 mL/min.; temperature 10°C. The fraction of lowest molar mass was obtained by ultrafiltration on 10000 cut-off membrane, concentration of the filtrate on a 1000 cut-off membrane, and chromatography on the Superdex 75 column.

Tubes corresponding to each fraction were pooled and evaporated to dryness in a centrifugal vacuum evaporation system (Savant).

Chromatographic Systems

Generally, for analytical purposes, a 150CV (Waters) system, composed of a liquid chromatograph equipped with an on-line single-capillary viscometer (SCV) and a differential refractometer detector (DRI) was used. Both detectors were exploited for the SEC-SCV viscometric characterization, whereas just the latter was utilized for standard SEC analyses. Millennium 2.15 software was used for data acquisition and conventional SEC and SEC-SCV data analysis. For semi-preparative purposes, a Waters 625 chromatograph was utilized.

Chromatographic Experimental Conditions

A ternary mobile phase, that proved suitable in the characterization of the previous conjugate polymer FCE 28068[5] was used: N,N-dimethylformamide (DMF, Aldrich) + 0.01 M LiBr (Sigma) + 0.05 M acetic acid (Carlo Erba Analyticals). The column set was composed of two Waters Styragel (HR4, HR3); flow rate was 0.8 mL/min; columns and detectors temperature were 50°C; the eluent was degassed with helium; toluene was used as flow marker.

Light Scattering

Measurements were performed with a multiangle laser light scattering photometer (MALLS) Dawn DSP-F (Wyatt). The MALLS instrument measures, through 15 photodiodes, the intensity of the scattered light over a broad range of angles (from 7° to 173° in methanol and F2 flow-cell). The light source is a vertically polarized 5 mW He-Ne laser, 632.8 nm of wavelenght. Data were analyzed by the Dawn 3.01 software from Wyatt. Details of MALLS hardware and software have been described elsewhere.[12] Instrument calibration was carried out with toluene as a standard, assuming a Rayleigh ratio value $R_\theta = 1.406 \times 10^{-5}$ cm^{-1}. The angular normalization of photodiodes was obtained using a concentrated solution of a narrow PEG standard (M = 12.6 Kg/mol; dispersity, D <1.04), assumed as isotropic scatterer. The used solvent for MALLS measurements was methanol (Baker) containing 0.05 M acetic acid. This solvent is incompatible with the usual SEC columns, therefore light scattering measurements were performed only in batch off-line mode. The refractive index increment, dn/dc, for PNU 166945 conjugate was determined by means of a Cromatix KMX-16 (Milton Roy) differential refractometer at 25°C in the above cited solvent.

On-Line Viscometry

Intrinsic viscosity, [η], was generally determined by the on-line SCV detector included in the Waters 150CV SEC-SCV system. For a control, some measurements of standards and polymer fractions, were carried out also in static off-line mode, with an Ubbelhode capillary viscometer. Details of the SCV detector, hardware and software, have been described elsewhere.[13] The concentration of the solutions was adjusted to obtain constant specific viscosity, $[\eta] \cdot c = 0.1$. On-line SCV detection is based on the concept of the universal calibration.[14] The universal calibration curve, third order polynomial fit, was generated by thirteen narrow PEO/PEG standards with the peak molar mass ranging from 600 to $8.5 \cdot 10^5$ g/mol. The description of the SEC-SCV multidetector system and of the related problems has previously been reported.[15]

The Mark-Houwink constants "k" and "a" of the narrow polymer fractions and of the PEO/PEG standards were calculated by least squares log-log fitting of [η] versus Mw average as obtained from MALLS in batch mode. For SEC standards, only the molar mass at peak apex (Mp) and dispersity were known. Still, for such narrow standards (D < 1.1), a lognormal distribution can be assumed, and thus Mw could be calculated using the following equation:

$$M_w = M_p \cdot \sqrt{D} \qquad (1)$$

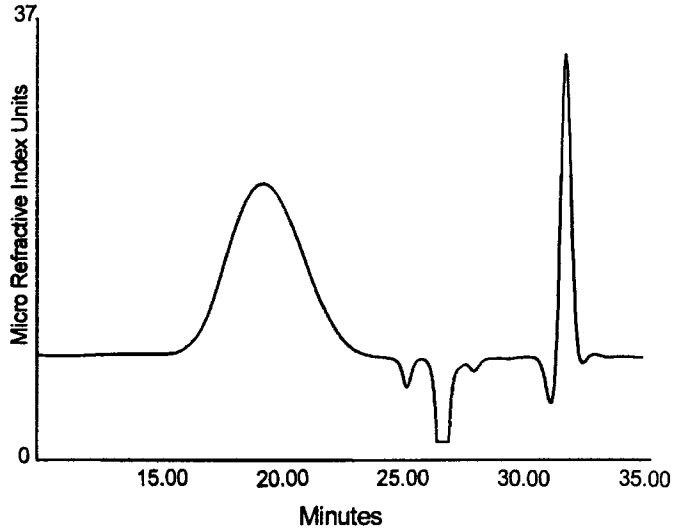

Figure 2. Chromatogram of a PNU 166945 sample (lot OF11361/68) as obtained in the experimental conditions by means of the refractive index detector. The narrow peak at about 32 min is due to toluene, the added flow-marker.

RESULTS AND DISCUSSION

A typical chromatogram of a PNU 166945 sample (lot OF11361/68), obtained in the experimental conditions is shown in Figure 2. As for the previous conjugate polymer, FCE 28068, the signal to noise ratio is quite high and the polymer peak is well separated from peaks of low molar mass contaminants. The peak at about 32 min is due to toluene used as a flow marker.

Viscometric Characterization of the PEO/PEG Narrow Standards

Seven PEO and six PEG were utilized to construct the universal calibration.[13] Standard Mp and dispersity values (as reported by the manufacturer) are summarized in Table 1, together with intrinsic viscosity values, as measured with the SEC-SCV on-line viscometer. These [η] values are in good agreement with the corresponding off-line measurements (not shown). The viscometric characterization of the PEO/PEG standards, in the used experimental conditions, was also reported in the previous work.[5] Actual [η] values are very close to the previous values.

Table 1

Viscometric Characterization of the PEO/PEG Narrrow Standards in SEC Mobile Phase at 50°C

No.	Mp g/mol	D	[η] dL/g
1	860,000	1.17	4.0465
2	570,000	1.10	3.1075
3	270,000	1.09	1.8681
4	160,000	1.07	1.2091
5	85,000	1.06	0.7669
6	45,000	1.07	0.4923
7	21,000	1.12	0.3056
8	12,600	1.04	0.2163
9	7,100	1.05	0.1548
10	4,100	1.05	0.0573
11	1,470	1.05	0.0573
12	960	1.03	0.0461
13	600	1.04	0.0378

Table 2

Mark-Houwink Equation Constants for PEG/PEO Narrow Standards and for PNU 166945 Conjugate Polymer, in SEC Mobile Phase at 50°C

Polymer	$k \cdot 10^4$ dL/g	a
PEO/PEG	4.33	0.666
PNU 166945	2.58	0.617

The Mark-Houwink constants for PEO/PEG standards, in the chosen mobile phase at 50 °C, were calculated from the data of Table 1, and are reported in Table 2. Figure 3 shows Mark-Houwink equation for PEO/PEG polymers. As we can see the relationship is linear also for low molar mass PEG standards.

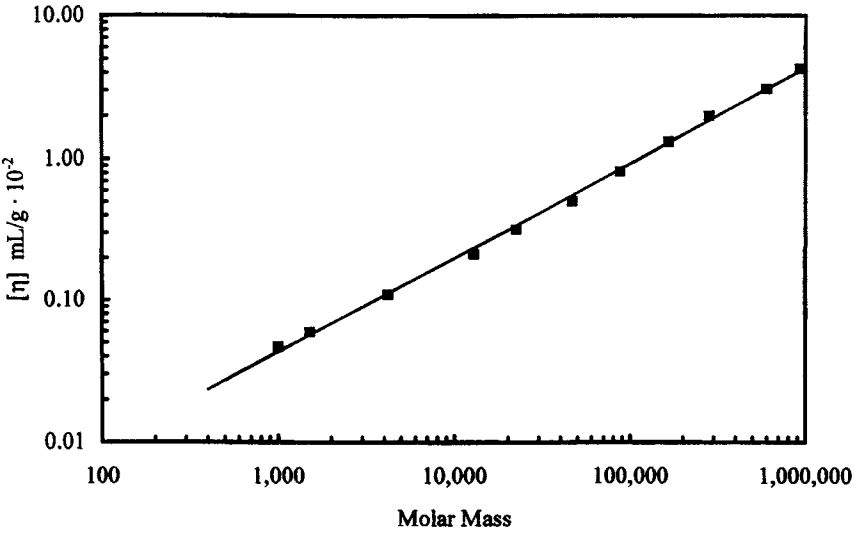

Figure 3. Derivation of Mark-Houwink equation parameters for PEO/PEG polymer, in SEC mobile phase at 50°C.

Table 3

Characterization of the PNU 166945 Fractions in SEC Mobile Phase at 50°C

No.	Mw g/mol	D	[η] dL/g
1	830,000	1.50	1.1401
2	322,000	1.61	0.6021
3	252,000	1.60	0.5119
4	188,500	1.58	0.4405
5	128,600	1.65	0.3426
6	77,300	1.33	0.2698
7	54,600	1.27	0.2081
8	38,500	1.30	0.1706
9	26,900	1.39	0.1361
10	18,600	1.18	0.1094
11	8,100	1.17	0.0722
12	6,200	1.19	0.0602
13	4,800	1.20	0.0507

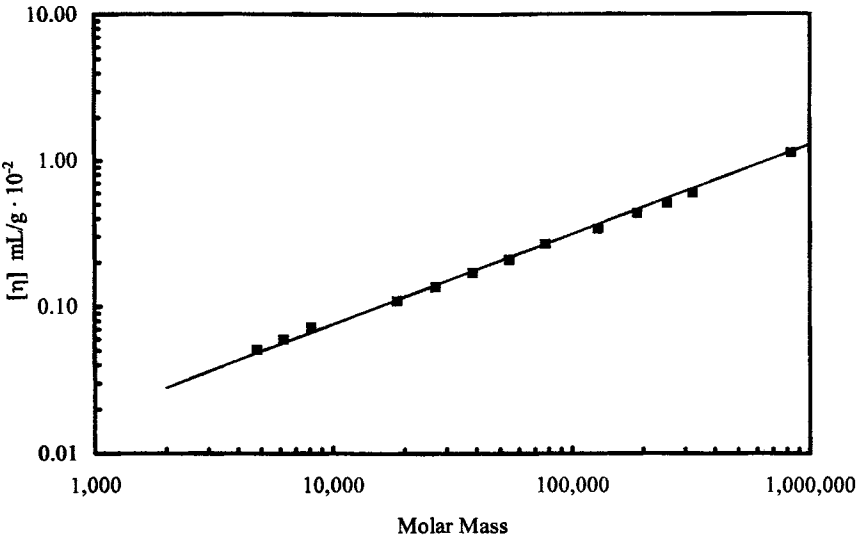

Figure 4. Derivation of Mark-Houwink equation parameters for PNU 166945 polymer fractions, in SEC mobile phase at 50°C.

Characterization of the PNU 166945 Fractions

The refractive index increment, dn/dc, for PNU 166945 polymer in methanol solvent at 25°C was 0.200 mL/g. This value is very close to the PHPMA homopolymer dn/dc value: 0.198 mL/g.

Every PNU 166945 fraction was accurately characterized as far as Mw, Mp, [η] and D are concerned. Corresponding values are reported in Table 3. In the same table, dispersity values are reported, as obtained with SEC and universal calibration. The thirteen PNU 166945 fractions show a broad molar mass range, but for some fractions the dispersity was not so narrow as we desired. Mw varied from $4.8 \cdot 10^3$ to $8.3 \cdot 10^5$ g/mol and dispersity varied from 1.17 to 1.65. In any case the MMD of the fractions was sufficiently narrow to make them adequate for a SEC calibration.

The Mark-Houwink constants for PNU 166945, in the chosen SEC mobile phase at 50°C, are reported in Table 2. The constants were obtained from [η] and Mw data reported in Table 3 discarding the three lowest molar mass fractions. Figure 4 shows the Mark-Houwink plot for PNU 166945 polymer fractions.

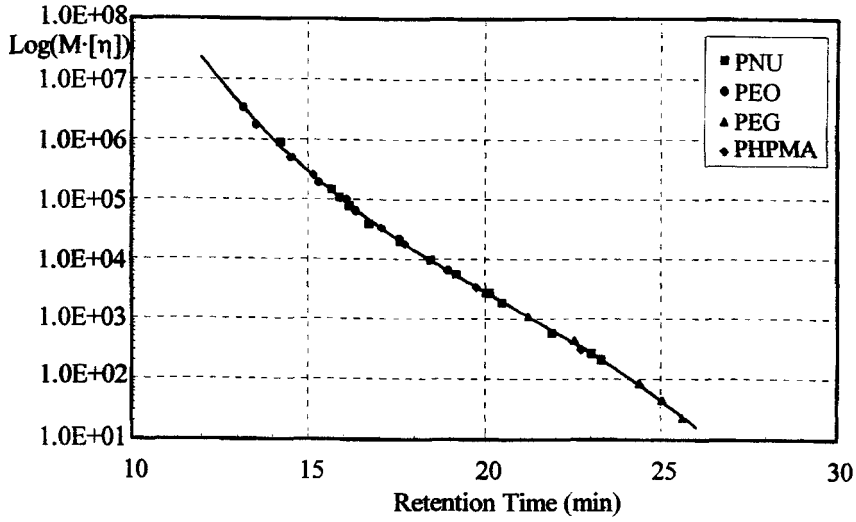

Figure 5. Test of the universal calibration for three sets of polymers: PEO/PEG narrow standards, PNU 166945 and PHPMA fractions (data relative to PHPMA fractions are derived from Ref. 5).

Check of Universal Calibration

The universal calibration holds if the experimental function Log(M·[η]) = f(V), where V is the elution volume, is common to the utilized polymers: PEO, PEG and PNU 166945. Accordingly, a true (not relative) MMD can be obtained, also when used standards are different from the analyzed polymer.

Figure 5 shows the experimental data of the above cited polymers, as obtained with the described SEC system, together with a third order polynomial data fitting (the calibration function). PNU 166945 fractions follow the universal calibration function (as calculated with the narrow PEO/PEG standards), which confirms that these polymers elute according to their hydrodynamic volume, and therefore they can be characterized by conventional SEC and universal calibration. It is worth noticing, with regard to the previous similar conjugate polymer FCE 28068, that the molar mass range of the thirteen fractions is much broader in the present case.

For a comparison, in Figure 5 we also report data of the six narrow MMD fractions of the PHPMA homopolymer. Molar mass and dispersity data for the six PHPMA fractions was reported in the previous paper.[5] PHPMA homopolymer also follows the universal calibration constructed with the PEO/PEG narrow standards.

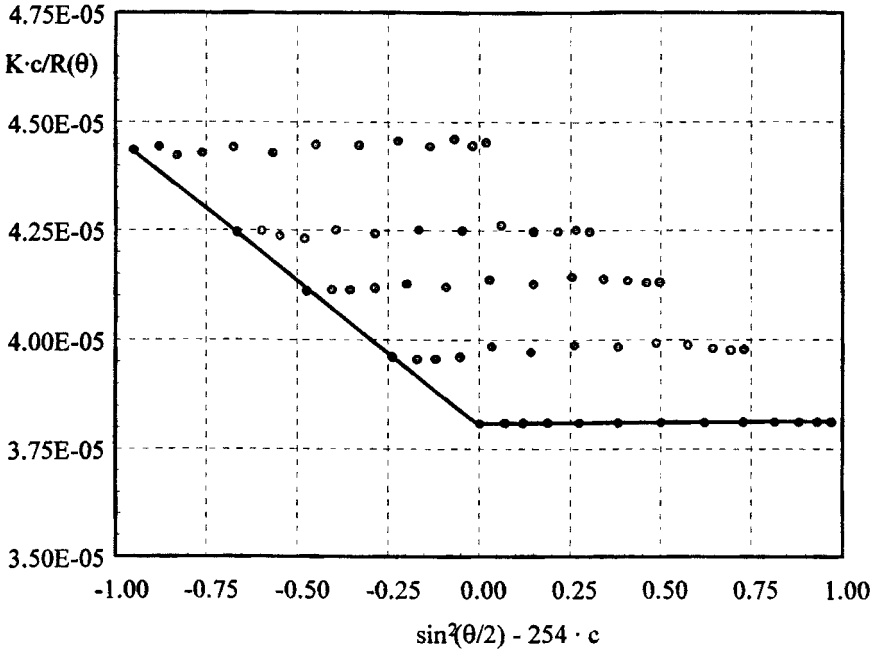

Figure 6. Zimm plot of a PNU 166945 sample (lot OF11361/01) in methanol and 0.05 M acetic acid at 25°C as obtained by MALLS in static off line mode.

Characterization of Unfractionated PNU 166945 Samples

Figure 6 shows the Zimm plot of an unfractionated PNU 166945 sample (lot OF21161/01). Mw of the sample was $2.65 \cdot 10^4$ g/mol; gyration radius $\langle s^2 \rangle_z^{1/2}$ was 5.7 nm; second virial coefficient A_2 was $6.26 \cdot 10^{-4}$ mol·cm^3·g^{-2}.

Table 4 shows the comparison between the Mw values of three PNU 166945 samples from three lots with different paclitaxel loading, as obtained from the off-line MALLS and from SEC and universal calibration. The difference between the values obtained with the two methods ranges from 4.3% to 5.1%. Table 4 also shows the comparison between [η] values obtained with three different methods: on-line SEC-SCV system, SEC and universal calibration method and, in one case, off-line Ubbelohde viscometer. The agreement between the on-line SEC-SCV and the off-line Ubbelhode viscometer is very good, lower than 1.2%. The difference between on-line SEC-SCV and SEC and universal calibration, ranges from 3.2% to 6.8%. A more exaustive characterization of the dilute-solution properties of PNU 166945 conjugate polymer is reported in Reference 16.

Table 4

Accuracy of the Characterization of Three PNU 166945 Samples

Sample	Paclitaxel % Weight	Mw(1) g/mol	Mw(2) g/mol	[η](3) dL/g	[η](4) dL/g	[η](2) dL/g	D(2)
OF11361/68	5.2	27,450	28,620	0.1293	0.1308	0.1350	1.65
OF11361/01	3.6	26,500	27,860	----	0.1224	0.1319	1.80
OF11361/82	0.0	26,100	27,300	----	0.1221	0.1315	1.63

(1) Off-line MALLS
(2) SEC and universal calibration
(3) Off-line Ubbelhode viscometer
(4) On-line SEC-SCV viscometery

SEC Measurement Accuracy

The difference between the true Mw values obtained by means of MALLS and the values obtained by SEC and universal calibration was lower than 5.1%. The difference for [η] values was lower than 6.8%. The agreement among true Mw and [η] values and data obtained by the described SEC and universal calibration method confirms the validity of universal calibration for PNU 166945 conjugate polymer.

SEC Measurement Reproducibility

SEC measurement reproducibility was determined by analyzing seven times, along eight months, a freshly prepared solution of a PNU 166945 sample (lot OF11361/68). Each time, a universal calibration curve with PEO/PEG standards was calculated. Average values of Mw and D are 27,700 and 1.56, respectively. The relative standard deviation (RSD% = 1.83 % for Mw and 1.03 % for dispersity) are well within the typical range for SEC determinations, and confirm the excellent reproducibility of the method.

CONCLUSIONS

From the considerable amount of data obtained in the described work with several techniques (SEC, SEC-viscometry, off-line MALLS, off-line viscometry) some significant conclusions can be driven. It is possible to characterize the PNU 166945 copolymer through conventional SEC in organic mobile phase after

calibration with commercial standards. Chromatographic separation of PNU 166945 in the specified experimental conditions is fundamentally based on its hydrodynamic volume, thus universal calibration operates. The SEC method is rapid: forty minutes are needed for one chromatogram with a set of just two high-efficiency columns (35-40,000 theoretical plates). PEO/PEG calibration gives Mw and [η] values close to those measured by light scattering in static mode and by off-line viscometry respectively. Finally, reproducibility of the described SEC method (RSD % < 2 %) is very high, which makes the method suitable for quality control.

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