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Molecular characterization of α,β-poly(asparthylhydrazide) a new synthetic polymer for biomedical applications

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Abstract

α,β-Poly(asparthylhydrazide) (PAHy) is a new synthetic polymer that exhibits interesting properties and is a candidate for biomedical applications. In this article the characterization of PAHy polymer by multi-angle laser light scattering (MALS) and single-capillary viscometer (SCV) detectors on-line to a size exclusion chromatography (SEC) system is reported. The SEC–MALS–SCV system furnishes exhaustive and consistent characterization of the PAHy polymer. Further, it is possible to characterize the PAHy polymer through conventional SEC and universal calibration. The universal calibration method gives intrinsic viscosity and dispersity very close to those measured by the absolute detectors; instead the weight-average molar mass is approximately 8% lower. This finding means that the chromatographic separation of PAHy polymer is fundamentally based on its hydrodynamic volume. By the SEC–MALS–SCV system the constants of the Mark–Houwink–Sakurada equation were also estimated.

Keywords: α,β-Poly(asparthylhydrazide); Molecular characterization; Drug carrier

1. Introduction

A great deal of interest has been aroused by the use of polymeric materials in the controlled drug delivery [1]. In this field such materials can be used to perform implants, hydrogels, micro- and nanoparticles, supramolecular micellar assemblies or polyelectrolytic complex containing drugs, polymeric drugs and polymeric carriers to which drugs can be linked by chemically and/or enzymatically hydrolyzable bonds [2,3]. In the latter case macromolecular prodrugs are obtained, which is able to deliver compound at the desired rate within the target compartment [4]. The major determinant of the pharmacokinetic characteristics in vivo of a macromolecular prodrug seems to be the macromolecule rather than the drug or targeting moiety [5]. Ideal macromolecular carriers should be easily synthesized at low cost, freely water-soluble, non-toxic, non-immunogenic and well characterized from the physico-chemical point of view [6]. Large importance in the distribution and elimination patterns of a macromolecular system is attributed to its physico-chemical properties, including molar mass distribution (MMD) and molecular size [5,6] and to its structural properties such as the presence of hydrophilic and/or hydrophobic portions, the incorporation of charged groups, the possible chemical dishomogeneities [7], etc. Also conformational properties of the macromolecules and their affinity with the medium can influence the biodistribution into the organism [4].

Studies of renal excretion and retention on animals performed on polyaspartamides at different molar mass demonstrating that clearance rate decreases with increasing molar mass [8]; but comparing with different materials it should seem better to relate the clearance of macromolecules to size rather than molar mass, since globular proteins and synthetic macromolecules of comparable molar mass have usually different sizes. Moreover, the flexible structure of the latter permits them to pass through glomerular pores by “end-up” motion allowing higher molar mass molecules to pass [7].

Unlike natural macromolecules, synthetic macromolecules are polydisperse. Therefore, it is important to characterize these macromolecules not only by their average molar mass but also by their MMD. To be eliminated from the organism, the whole MMD must be under the threshold for glomerular filtration. Studies on the biological fate of some preparations of [^{14}C] Polyvinylpyrrolidone with the same molar mass but different polydispersities provided evidence for the importance of the MMD [9]. The preparation with higher polydispersity (containing a fraction of macromolecules with higher molar mass) was found to be

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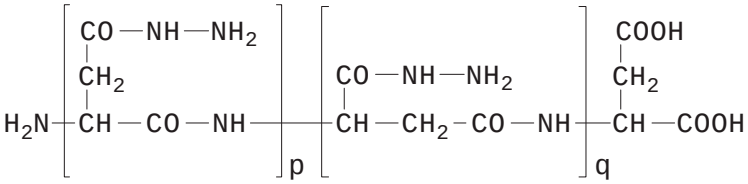

Fig. 1. Structure of the PAHy polymer.

retained in the organs for a much longer time than the less polydisperse preparation [9].

α,β-poly(asparthylhydrazide) (PAHy) (Fig. 1), is a water-soluble synthetic polymer, with a protein-like structure [10,11], obtained by a simple reaction of hydrazine with a polysuccinimide that is easily prepared by thermal polycondensation of D,L-aspartic acid [12]. Recent studies demonstrated the excellent toxicological and pharmacological properties of PAHy and the possibility to propose this polymer as plasma expander and drug carrier [11].

In the last few years a systematic study of the physicochemical properties of PAHy has been carried out [13,15]. Recently, an exhaustive conformational analysis in aqueous solution by means of the small-angle X-ray scattering (SAXS) technique has been presented [15]. The present study completes the results obtained by the SAXS characterization. We have investigated the molecular properties in aqueous solution of the PAHy polymer by means of Size Exclusion Chromatography (SEC), multi-angle laser light scattering (MALS) and single-capillary viscometer (SCV). In particular, we have used the absolute detectors, MALS and SCV, on-line to the SEC system. The method provides, without calibration, the MMD of the polymer, the intrinsic viscosity distribution, the Mark–Houwink–Sakurada power law and the dimensions of the macromolecules.

2. Experimental

2.1. PAHy synthesis

PAHy samples were prepared by the reaction of polysuccinimide with hydrazine in *N*,*N*-dimethylformamide solution and purified according to a procedure already described [13,14]. Spectroscopic data (FT-IR and NMR) were in agreement with the literature values [13,14].

2.2. Materials

Seven poly(ethylene oxide) (PEO) narrow MMD standards were obtained from Toyo Soda (Tokyo, Japan). Seven poly(ethylene glycol) (PEG) narrow MMD standards were obtained from Polymer Laboratories (Shropshire, UK). Bovine Serum Albumin (BSA) was obtained from Sigma (St. Louis, USA). Water solvent was MilliQ grade Millipore (Bedford, USA). All other chemicals were of analytical grade.

2.3. Methods

2.3.1. Chromatographic system

The MMD of the PAHy samples was obtained with an original chromatographic multidetector SEC system. The system was obtained assembling a pulse-free pump and three on-line detectors. The system consisted of: Alliance 2690 separations module, single-capillary viscometer (SCV), differential refractometer (DRI) from Waters (Milford, MA, USA) and an additional MALS photometer from Wyatt (S. Barbara, CA, USA). The description and the performance of this new multidetector SEC system has been described in detail elsewhere [16,17] and will not be reported herein. The columns set was composed of a precolumn and two Ultrahydrogel columns (1000 and 250 Å of pore size) from Waters. The experimental conditions consisted of: 0.1 M $NaNO_3$ + phosphate buffer pH 7.8 as mobile phase, 35°C of temperature, 0.8 ml/min of flow rate, 200 μl of injection volume.

2.3.2. Light scattering

The MALS Dawn DSP-F photometer, 632.8 nm of wavelength, measures the intensity of the scattered light at 18 fixed angular locations ranging, in the mobile phase, from 8.9 to 171.1°. Data acquisition and analysis software was ASTRA 4.50 from Wyatt. MALS hardware and analysis software have been described in detail elsewhere [18]. It is well known that the MALS detector measures, from the intensity of the scattering, the molar mass of the polymer. Further, from the angular variation of the scattering, the MALS detector measures the dimension of the molecules: the root mean square radius $\langle s^2\rangle^{1/2}$ will be denoted in short hereafter as gyration radius. The calibration constant was calculated using toluene as standard assuming a Rayleigh factor of $1.406 \times 10^{-5}\,cm^{-1}$. The photodiodes angular normalization was made by measuring the scattering intensity of a BSA globular protein in the mobile phase assumed to act as an isotropic scatterer. The experimental methodology to get reliable results from the MALS detector have been described previously [19,20]. The light scattering characterization has been performed both in the static off-line mode, in short denoted as MALS, to measure the true weight-average molar mass (M_w) and in the on-line mode to the SEC system, in short denoted as SEC–MALS, to determine the whole MMD and the dimension of the PAHy molecules.

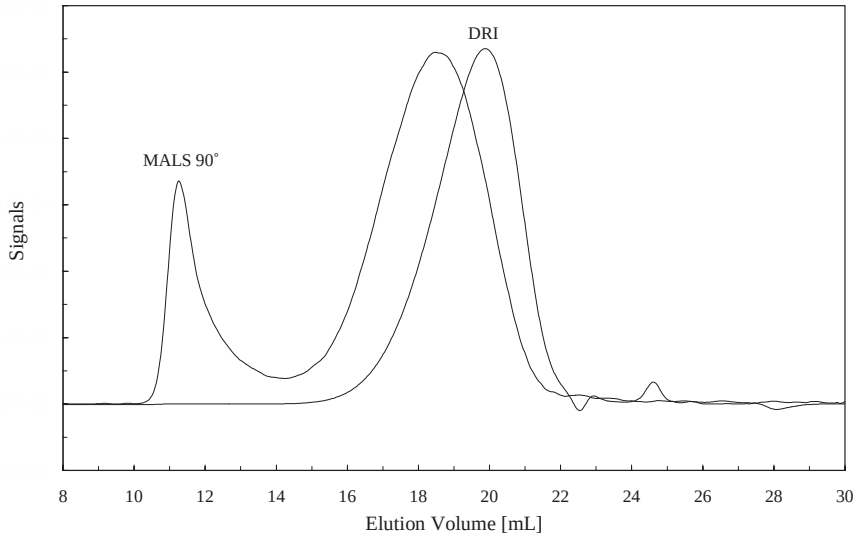

Fig. 2. Signals, MALS 90° and DRI, of a PAHy sample in 0.1 M $NaNO_3$ mobile phase.

2.3.3. dn/dc

The specific refractive index increment, *d*n/*d*c, of the PAHy polymer with respect to the mobile phase at 25°C was measured by a KMX-16 differential refractometer from LDC Milton Roy (Riviera Beach, USA). The *d*n/*d*c value was 0.190 ml/g.

2.3.4. Viscometry

SCV data acquisition and analysis software was MILLENNIUM 2.15 from Waters. Details of the SCV analysis software have been described elsewhere [21–23]. The signal of the viscometer detector depends on the intrinsic viscosity and on the concentration of the solution. Hence to obtain constant signal-to-noise ratio the concentration of the samples has been adjusted so that $[\eta]\cdot c = 0.1$. On-line SCV detection is based on the concept of the universal calibration [24]. The universal calibration curve, polynomial third-order fit, was generated by fourteen PEO/PEG narrow MMD standards with the peak molar mass (M_p) ranging from 106 to 8.6×10^5 g/mol.

The intrinsic viscosity of a PAHy sample was also measured in static off-line mode by a conventional micro-Ubbelohde viscometer, in short denoted as Visc. Off-line $[\eta]$ value has been used as reference for SCV on-line value. Off-line viscosity data analysis has been performed by the usual Huggins and Kraemer relationship.

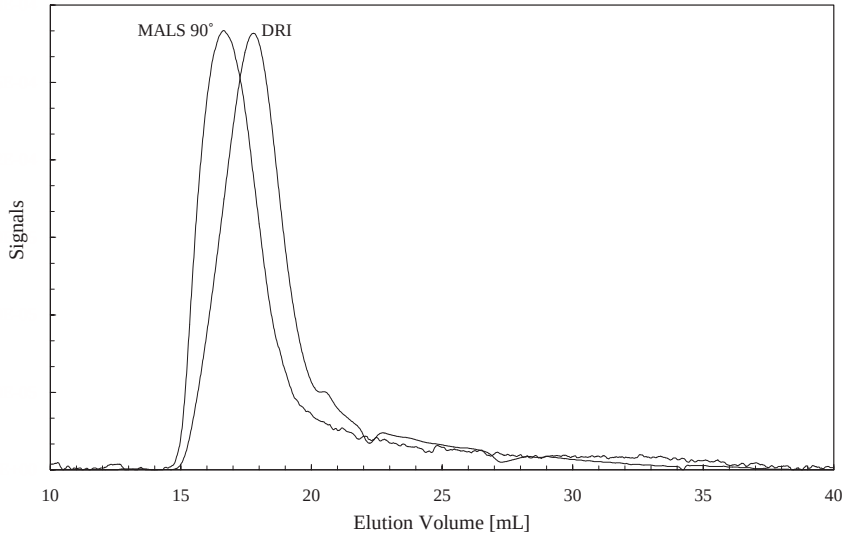

Fig. 3. Signals, MALS 90° and DRI, of a PAHy sample in 0.1 M $NaNO_3$ + TFA pH 3.0 mobile phase.

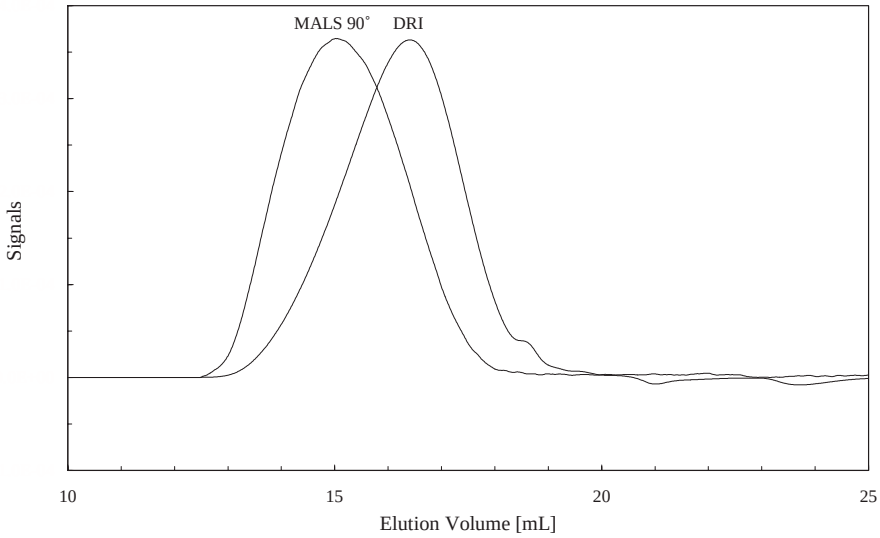

Fig. 4. Signals, MALS 90° and DRI, of a PAHy sample in 0.1 M $NaNO_3$ + phosphate buffer pH 7.8 mobile phase.

2.3.5. Interdetectors delay volume

In a multi-detector SEC system accurate values of the interdetectors delay volume between the absolute detectors, MALS and SCV, and the concentration detector, DRI, must be accounted for. Local properties, M_i and $[\eta]_i$, at each retention volume are very sensitive to a wrong superimposition of the MALS, SCV and concentration signals. The value of the MALS–DRI interdetectors delay volume, used in the data reduction software, was 380 μl. The value of the SCV–DRI interdetectors delay volume was 80 μl. The procedure to determine the interdetectors delay volume have been described previously [17,20].

3. Results and discussion

3.1. SEC mobile phase

The fractionation of the PAHy polymer on SEC columns presents many problems. PAHy samples are freely soluble in aqueous solvent; on the contrary, they are not soluble in organic solvents. If columns set, ionic strength and pH of the aqueous mobile phase are not optmized the chromatograms show aggregation and/or long tails. Fig. 2 shows the signals, MALS 90° and DRI, obtained with a PAHy sample in 0.1 M $NaNO_3$ mobile phase. The MALS detector is very sensitive to high molar mass aggregates. In the figure. we can see that the first peak of the chromatogram, approximately 11.2 ml of elution volume, of the MALS detector is very high. On the contrary, the relative signal of the DRI concentration detector is imperceptible. This means that very low amount of ultra-high molar mass aggregates are present. Instead, Fig. 3 shows the chromatogram of the PAHy sample with a long tail. In this case the mobile phase was 0.1 M $NaNO_3$+ trifluoroacetic acid pH 3. In this mobile phase we can see a strong interaction of the PAHy macromolecules with the packing of the Ultrahydrogel columns. Non-size exclusion fractionation is also a problem. We have used absolute on-line SEC detectors, MALS and SCV, that measured directly molar mass and intrinsic viscosity of every slice, fraction, of the chromatogram. In theory the three detectors system allows moderate non-size exclusion fractionation. In any case it is better to optimize the experimental conditions so as to obtain a steric fractionation without large aggregates. Fig. 4 shows the signals when ionic strength and pH of the aqueous mobile phase were optmized. In this case the mobile phase was 0.1 M $NaNO_3$+ phosphate buffer pH 7.8. We can see that the signal-to-noise ratio was very good, the polymer peak was symmetrical and well separated from the impurity (system) peaks.

Table 1
Characterization of the PAHy_1 sample by five different methods

MALS	M_w[a] =	$\langle s^2\rangle_z^{1/2}$ (nm)	A_2 (mol ml g^{-2})
	21 120	—	-3.74×10^{-5}
Visc	M_v[a] =	$[\eta]$ (dl/g)	
	18 080	0.069	
SEC–MALS	M_w[a] =	$\langle s^2\rangle_z^{1/2}$ (nm)	*D*
	21 240	—	2.2
SEC–SCV	M_w[a] =	$[\eta]$ (dl/g)	*D*
	20 050	0.067	2.1
SEC–UC	M_w[a] =	$[\eta]$ (dl/g)	*D*
	19 280	0.066	2.1

[a] M_w is in g/mol.

3.2. SEC–MALS–SCV

A summary of the results for the PAHy_1 sample obtained using five characterization methods is listed in Table 1. First two methods, MALS and Visc, were the

Table 2
Summarized results of the characterization of three PAHy samples by three SEC methods

Sample	SEC–MALS			SEC–SCV			SEC–UC		
	M_w g/mol	$\langle s^2\rangle_z^{1/2}$ (nm)	D	M_w (g/mol)	$[\eta]$ (dl/g)	D	M_w (g/mol)	$[\eta]$ (dl/g)	D
PAHy_1	21 240	—	2.2	20 050	0.067	2.1	19 280	0.066	2.1
PAHy_2	12 820	—	2.3	14 660	0.056	2.2	14 285	0.057	2.0
PAHy_3	8050	—	2.4	12 400	0.050	2.2	11 800	0.051	2.2

classical static off-line light scattering and viscometry modes. Last three methods were on-line to the SEC system. SEC–MALS was the dual detector system MALS and DRI. SEC–SCV was the dual detector system SCV and DRI. Finally, only for comparison, SEC–UC was the conventional SEC and universal calibration method using narrow MMD PEO/PEG standards as calibrant.

The weight-average molar mass, M_w, of the PAHy_1 sample was 21 120 g/mol by MALS, 21 240 g/mol by SEC–MALS and 20 050 g/mol by SEC–SCV. The agreement between the results of the three methods was good. The difference, with regard to the M_w average, was lower than 5.6%. The intrinsic viscosity, $[\eta]$, of the PAHy_1 sample was 0.069 dl/g by the off-line viscometer, Visc, and 0.067 dl/g by the on-line viscometer, SEC–SCV. Again, despite the low value of $[\eta]$, the agreement between the results of the two methods was very good. The dispersity index, D, was 2.2 and 2.1 by SEC–MALS and SEC–SCV respectively. Substantially the off-line results, M_w and $[\eta]$, confirm the results obtained with the on-line methods. Besides the results obtained with the two on-line methods were congruent.

Table 2 shows a comparison between the results of three PAHy samples as obtained from SEC–MALS and SEC–SCV. For comparison Fig. 5 shows the differential MMD of the three PAHy samples as obtained from the SEC–MALS system. The agreement between SEC–MALS and SEC–SCV results was very good. There is a notable exception, the M_w value of the PAHy_3 sample. The M_w average obtained with the on-line SEC–MALS method is notably lower than the SEC–SCV value: respectively, 8050 and 12 400 g/mol. To partial explanation of this anomalous result we remember that the molar mass of the sample is quite low, with respect to the sensitivity of the light scattering detector. Besides the dn/dc value, when the molar mass of the polymer is approximately lower than 20 kg/mol is not constant. Probably the SEC–SCV M_w result is more reliable. Further, the intrinsic viscosity value confirms the SEC–SCV M_w result.

Finally, the second virial coefficient, A_2, was -3.74×10^{-5} mol ml g^{-2}. The negative low value of A_2 confirms that the mobile phase was a poor solvent for the PAHy polymer. Of course, the low value of the molar mass of the PAHy sample should also be taken into account.

3.3. SEC–universal calibration

Surprisingly, the agreement between SEC–universal calibration (SEC–UC) results and SEC–MALS–SCV results was quite good. $[\eta]$ and D data of the three PAHy samples, reported in the Table 2, substantially agree. There is a little difference, lower than 8%, only for the M_w values. This

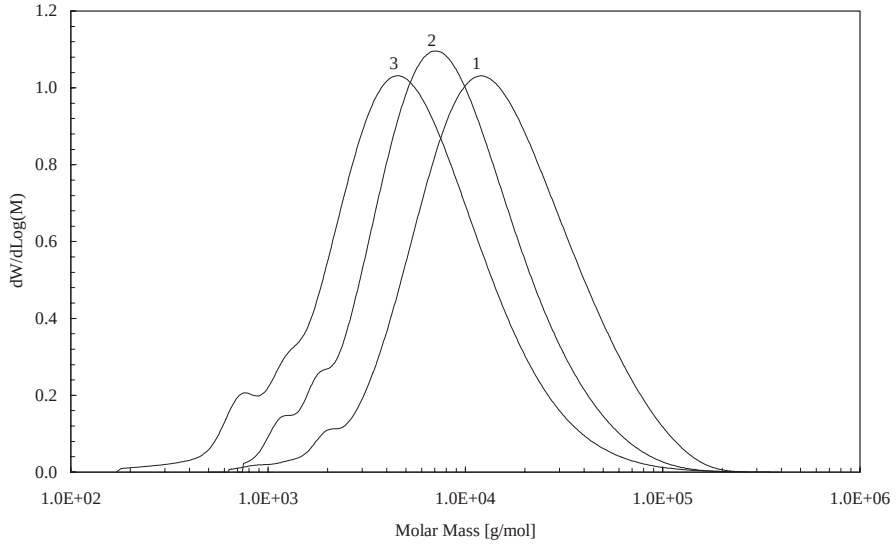

Fig. 5. Comparison of the differential molar mass distributions of three PAHy samples.

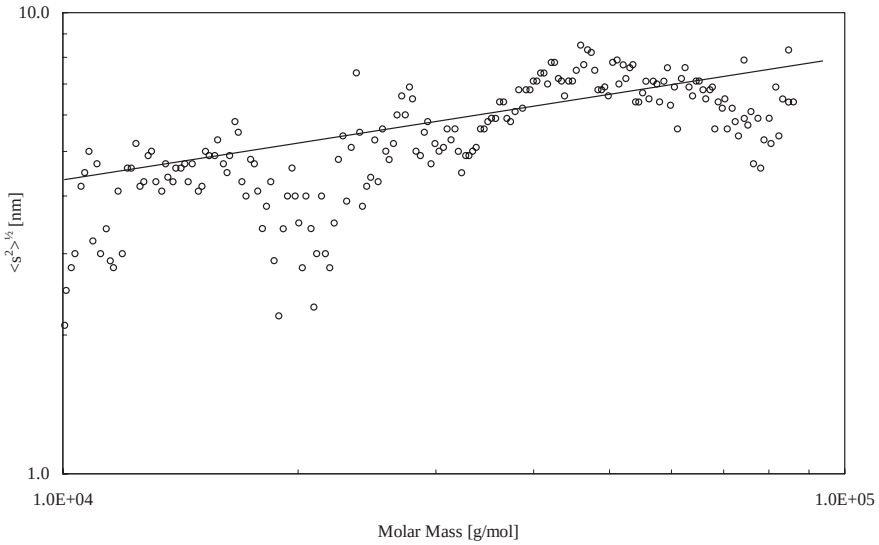

Fig. 6. $< s^2 >^{1/2}$ experimental data of the PAHy_1 sample from the SEC–MALS system.

finding means that the chromatographic separation of PAHy polymer, in the specified experimental conditions, is fundamentally based on its hydrodynamic volume. Considering the simplicity of the SEC–UC method as regard to the SEC–MALS–SCV method this result is meaningful.

3.4. Dimension of the macromolecules

Measurement of gyration radius by MALS requires that the angular dependence is experimentally measurable. The minimum gyration radius value measurable with the He–Ne laser in aqueous solvent is about 8–10 nm. The gyration radius of the PAHy_1 sample estimated with the SAXS method was 2.1 nm [15]. Hence, in this case the accuracy of the MALS technique for the gyration radius is poor because the dimensions were lower than the measurable limit of the instrument. With regard to the dimension of the PAHy macromolecules we can show only semi-quantitative results. Assuming ideal SEC fractionation, every slice of a SEC–MALS chromatogram could be considered homogeneous in molar mass and in dimensions. Besides increasing the concentration of the sample, to obtain better signal-to-noise ratio, we could extend toward lower values the measurable limit of the gyration radius. Fig. 6 shows the experimental gyration radius data for the PAHy_1 sample from the SEC–MALS system. We can see that the dimensions of the PAHy macromolecules in the experimental molar mass range, ~10–100 kg/mol, roughly range from

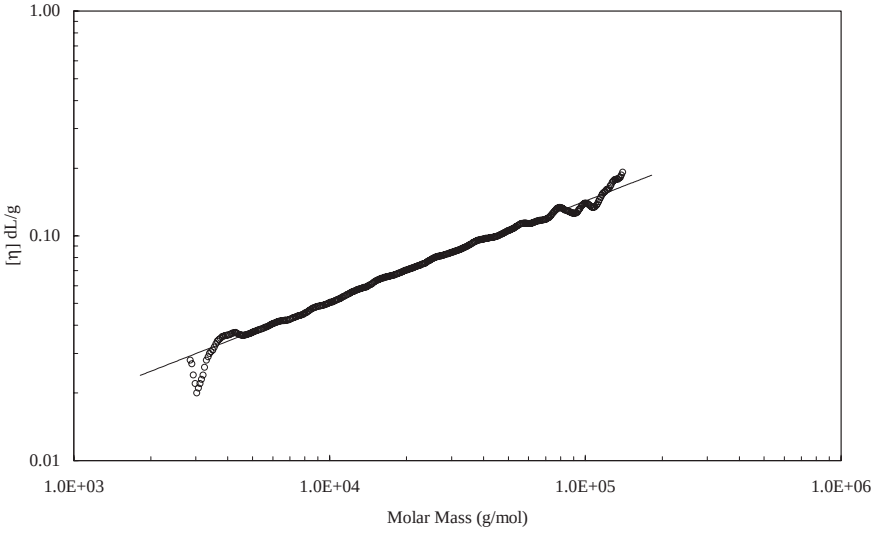

Fig. 7. $[\eta] = f(M)$ power law for the PAHy polymer in the mobile phase at 35°C.

Table 3
Constants of the MHS equation for three PAHy samples by SEC–SCV

Sample	$k \times 10^4$ dl/g	a
PAHy_1	3.75	0.532
PAHy_2	3.75	0.532
PAHy_3	2.70	0.500

2 to 8 nm. Obviously, the gyration radius data in this range are scattered.

3.5. Mark–Houwink–Sakurada plot

We were interested to estimate the parameters of the $[\eta] = f(M)$ power law, Mark–Houwink–Sakurada (MHS) plot: $[\eta] = kM^a$. The molar mass and the intrinsic viscosity of the PAHy samples were quite low, respectively lower than 21 kg/mol and 0.07 dl/g. Hence, it was impossible to obtain an adequate number of narrow MMD fractions, to span a wide range of molar mass, to estimate the parameters of the MHS equation in the usual mode. Hence, we have chosen to estimate the parameters of the MHS equation from a single broad MMD PAHy sample using the on-line SCV detector. The estimation of reliable values of the parameters of the MHS equation from a single broad MMD sample by the commercially available SEC–SCV system presents many problems [21,22]. For this reason we have used the new SEC–SCV system.

Fig. 7 shows the experimental $[\eta] = f(M)$ power law, MHS plot, for the PAHy polymer in the mobile phase at 35°C. The experimental data has obtained from the SEC–SCV system using the PAHy_1 sample. Despite the low molar mass of the samples the signal-to-noise ratio was quite good and with the exception of the extremities of the plot the $[\eta]$ values were accurate. Table 3 reports the constants, "k" and "a", of the MHS equation as obtained from the SEC–SCV system using the three PAHy samples. Except for the PAHy_3 sample, where the molar mass is very low, the MHS constants, for the PAHy polymer in 0.1 M $NaNO_3$ + phosphate buffer pH 7.8 at 35°C, were: $k = 3.75 \times 10^{-4}$ and $a = 0.532$. Therefore in these experimental conditions the MHS equation for PAHy can be expressed as:

$$[\eta] = 0.000375M^{0.532}.$$

SAXS characterization found that the PAHy polymer in aqueous solution assumes semi-rigid conformation [15]. The slope of the MHS equation for the PAHy polymer, 0.532, is not high. However, we have to consider two problems. First, the used solvent was poor; the value of the second virial coefficient was negative. Second, the molar mass of the samples was quite low and in these conditions the excluded volume substantially does not operate. Hence, our results are consistent with the conformational SAXS results for the PAHy polymer.

4. Conclusions

From the considerable amount of data obtained in the present work with several techniques we have described a complete and consistent picture of the molecular properties of the new water soluble PAHy polymer. From these results some significant conclusions can be made. The SEC–MALS–SCV system furnishes an exhaustive and consistent molecular characterization of the PAHy polymer. Further, it is possible to characterize the PAHy polymer through conventional SEC and universal calibration method using commercial PEO/PEG standards. The PEO/PEG universal calibration gives $[\eta]$ and D values very close to those measured by the on-line absolute detectors. The M_w value by SEC–UC method was a little different from the value by SEC–MALS–SCV methods. However, this difference was lower than 8%. This finding means that the chromatographic separation of PAHy polymer is fundamentally based on its hydrodynamic volume. By the SEC–MALS system we have obtained an approximate estimation of the dimension of the PAHy molecules. By the SEC–SCV system we have also estimated the constants of the MHS equation in 0.1 M $NaNO_3$ + phosphate buffer pH 7.8 at 35°C.

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