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Molecular characterization of α,β-poly[(*N*-hydroxyethyl)-DL–aspartamide] by light scattering and viscometry studies

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Abstract

α,β-poly[(*N*-hydroxyethyl)-DL-aspartamide] (PHEA) is a new synthetic polymer which is of interest in biomedical applications. In this paper, the molecular characterization of PHEA by multi-angle laser light scattering and viscometry off-line and on-line to a size exclusion chromatography system is reported. These techniques furnish an exhaustive and consistent characterization of the PHEA polymer. The fractionation of the PHEA macromolecules was relatively simple. Using an aqueous mobile phase of medium ionic strength, the elution was substantially regular and the macromolecules were not aggregate. The molar mass M of four PHEA samples approximately ranges from 46 to 53 K g/mol, the intrinsic viscosity $[\eta]$ ranges from 0.22 to 0.26 dl/g and the gyration radius R_g is also estimated. Both the slopes of the $[\eta] = f(M)$ and $R_g f(M)$ power laws prove that PHEA macromolecules in aqueous solution are flexible coils. Besides, the second virial coefficient value proves that the used mobile phase is a good solvent for PHEA. The molecular characterization of PHEA was also performed through conventional SEC and universal calibration and the estimated molar mass was substantially higher than that evaluated by absolute methods.

Keywords: α,β-Poly[(*N*-hydroxyethyl)-DL-aspartamide]; Drug carrier; Size exclusion chromatography

1. Introduction

A widespread consensus exists that the success of polymeric materials in controlled drug delivery systems is mainly due to the properties of used macromolecular carriers [1,2]. Therefore, to characterize both from the physico-chemical and toxicological point of view, a polymeric material is becoming more important and is noticed both by the academic and industrial world. A complete and exhaustive characterization study of polymeric materials includes, besides the determination of the molar mass, molar mass distribution (MMD) and molecular size as well as conformational properties and the affinity of polymers with various media [3,4]. All these aspects in effect play a fundamental role in determining either in the chemical field the reactivity of materials or in the biological field the fate of these materials in the body when they are administered as supports in drug delivery systems [5,6].

On the other hand, recently much progress has been made in the field of physico-chemical characterization of polymers as apparatus and methodologies, and the assembling of many techniques together can in general give more congruent information than the same single and separate methodologies. This is the reason for using multi-angle laser light scattering (MALS) and a viscometer as on-line detectors to a size exclusion chromatography (SEC) system [7–10]. The method has already been proposed for other polymers and among them for α,β-polyasparthydrazide (PAHy), a new protein-like structure water-soluble polymer [11] can successfully provide without calibration the MMD, the intrinsic viscosity and the dimension of the macromolecules.

α,β-poly[(*N*-hydroxyethyl)-DL-aspartamide] (PHEA) [12] (Fig. 1), is a water-soluble synthetic polymer, with a polyaminoacidic structure, obtained by a simple reaction of ethanolamine with polysuccinimide (PSI), which is easily prepared by thermal polycondensation of D,L-aspartic acid [12]. Its favourable toxicological properties, i.e. lack of toxicity, antigenicity and immunogenicity, allowed its proposition as a plasma expander in the biomedical field [13]. Recent studies showed the possibility of proposing this polymer as a drug carrier in the synthesis of

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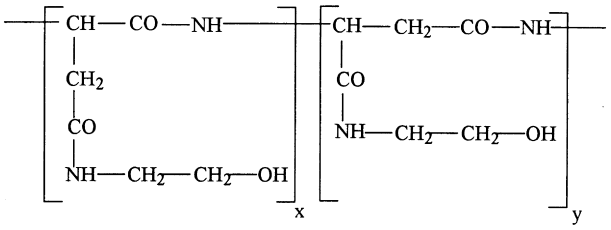

Fig. 1. Structure of the PHEA polymer.

macromolecular prodrugs of antiviral agents such as acyclovir and zidovudine [14,15]. In addition it was also used after proper derivatization as the parent material to obtain cross-linked systems by UV or γ-irradiation [16,17].

In the last few years, systematic studies of the physico-chemical properties of PHEA were able to offer more useful information to rationalize the behaviour in vivo of the polymer [18–21]. Recent small-angle X-ray scattering (SAXS) experiments showed that in aqueous solution PHEA can be represented by a random coil conformation in which the flexibility of the backbone due to the presence of methylenic groups and the wide interactions of the side-chains with water molecules play a fundamental role in the high solubility of this polymer in water and in several other polar solvents (e.g. dimethylformamide) [21,22].

In this paper we present an exhaustive molecular characterization of PHEA by using light scattering and viscometry techniques both off-line and on-line to a SEC system. Owing to the common parent polymer (PSI) and a very similar polymeric backbone between polyaspartamide and polyasparthydrazide, a comparison of the molecular properties of PHEA and PAHy polymers is presented.

2. Experimental

2.1. PHEA synthesis

Four PHEA batches ($PHEA_1$, $PHEA_2$, $PHEA_3$, $PHEA_4$) were synthesised by the reaction of four batches of polysuccinimide with ethanolamine and purified according to a procedure reported elsewhere [12]. Spectroscopic data (FT-IR and NMR) were in agreement with the literature values [14].

2.2. Materials

Poly(ethylene oxide) (PEO) narrow MMD standards were obtained from Showa Denko (Tokyo, Japan). Poly(ethylene glycol) (PEG) narrow MMD standards were obtained from Polymer Laboratories (Shropshire, UK). Bovine serum albumin (BSA) was obtained from Sigma (S. Louis, USA). Water was MilliQ grade Millipore (Bedford, USA). All other chemicals were of analytical grade.

2.3. Methods

The molecular characterization of the PHEA samples was performed by a multi-detector SEC system. The system consisted of an Alliance 2690 separations module, a single capillary viscometer (SCV), a differential refractometer (DRI) from Waters (Milford, MA, USA) and an additional MALS photometer from Wyatt (S. Barbara, CA, USA). Because the SEC system used in the characterization of PHEA was identical to the system used in the characterization of PAHy [11] the description will be not reported here. The columns set was composed of a precolumn and three

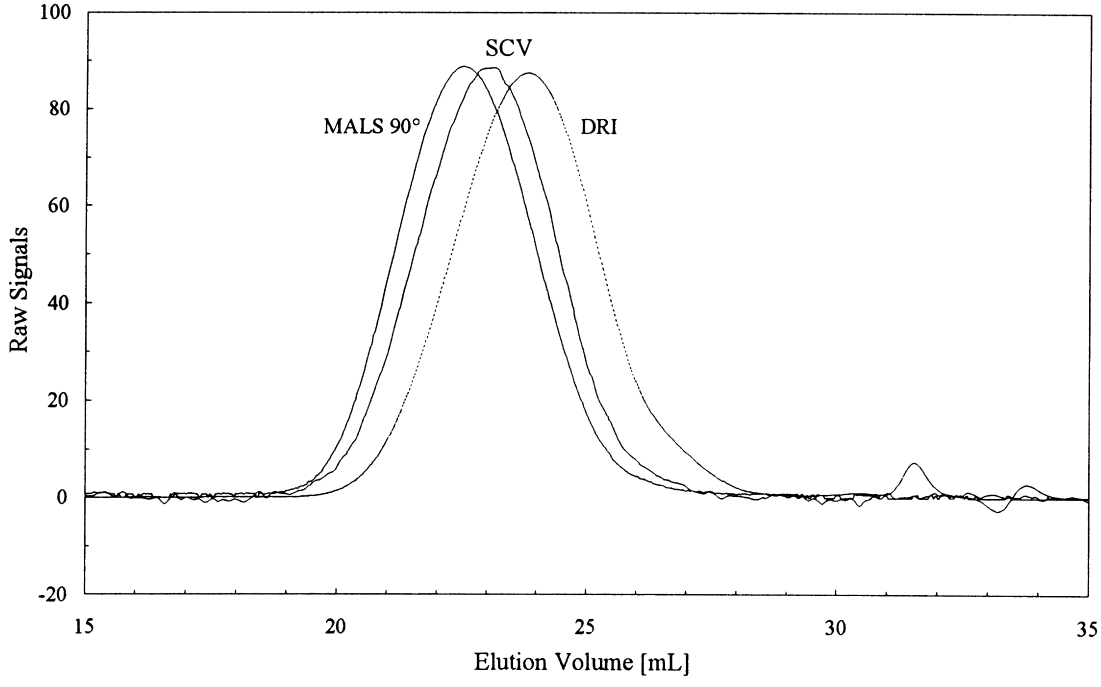

Fig. 2. Signals (MALS 90°, SCV and DRI) of the $PHEA_3$ sample.

Table 1
Summary of the characterization of the PHEA samples

Sample	MALS			SEC–MALS		SEC–SCV [η] (dl/g)
	M_w (g/mol)	A_2 (mol ml g^{-2})	R_g (nm)	M_w (g/mol)	D	
$PHEA_1$	53,500	3.6×10^{-4}	8.3	53,020	1.8	0.26_1
$PHEA_2$				52,480	1.8	0.26_2
$PHEA_3$				46,280	1.7	0.22_4
$PHEA_4$				47,250	1.8	0.22_4

Ultrahydrogel columns (1000, 500 and 120 Å of pore size) from Waters. The experimental conditions consisted of: 0.2 M NaCl + 0.l M Tris pH 8 as mobile phase, 35°C as temperature, 0.8 ml/min as flow rate and 300 μl as injection volume.

The calibration constant of the MALS photometer was calculated using Toluene as standard assuming a Rayleigh Factor $R(\theta) = 1.406 \times 10^{-5}$ cm^{-1}. The photodiodes angular normalization was performed by measuring the scattering intensity of a BSA globular protein in the mobile phase assumed to act as an isotropic scatterer. The light scattering characterization has been performed both in the static off-line mode, in short denoted as MALS, to measure the weight-average molar mass M_w and the second virial coefficient A_2 and in the on-line mode to the SEC system, in short denoted as SEC–MALS, to determine the MMD and the dimension of the molecules the root mean square radius $\langle s^2 \rangle^{1/2}$, in short hereafter denoted as the gyration radius R_g. Further, the on-line SCV detector measured the intrinsic viscosity at each elution volume, $[\eta]_i$. Virtually, at each elution volume, after the fractionation on the SEC columns, the SEC–MALS–SCV system provided M_i, R_{g_i}, and $[\eta]_i$ of the macromolecules.

2.4. d*n*/d*c*

The specific refractive index increment, d*n*/d*c*, for PHEA with respect to the mobile phase at 25°C was measured by a KMX-16 differential refractometer from LDC Milton Roy (Riviera Beach, FL, USA). The d*n*/d*c* value for PHEA was 0.169 ml/g.

3. Results and discussion

The fractionation of PHEA on some Ultrahydrogel SEC columns was quite simple. Using an aqueous mobile phase such as 0.1 M $NaN0_3$ of medium ionic strength the polymer peak was symmetrical, well separated from the impurity peaks, without meaningful aggregates and tails. Fig. 2 shows the raw signals, in 0.1 M $NaN0_3$ mobile phase, of MALS (90° photodiode), SCV and DRI on-line detectors. We can see that all the

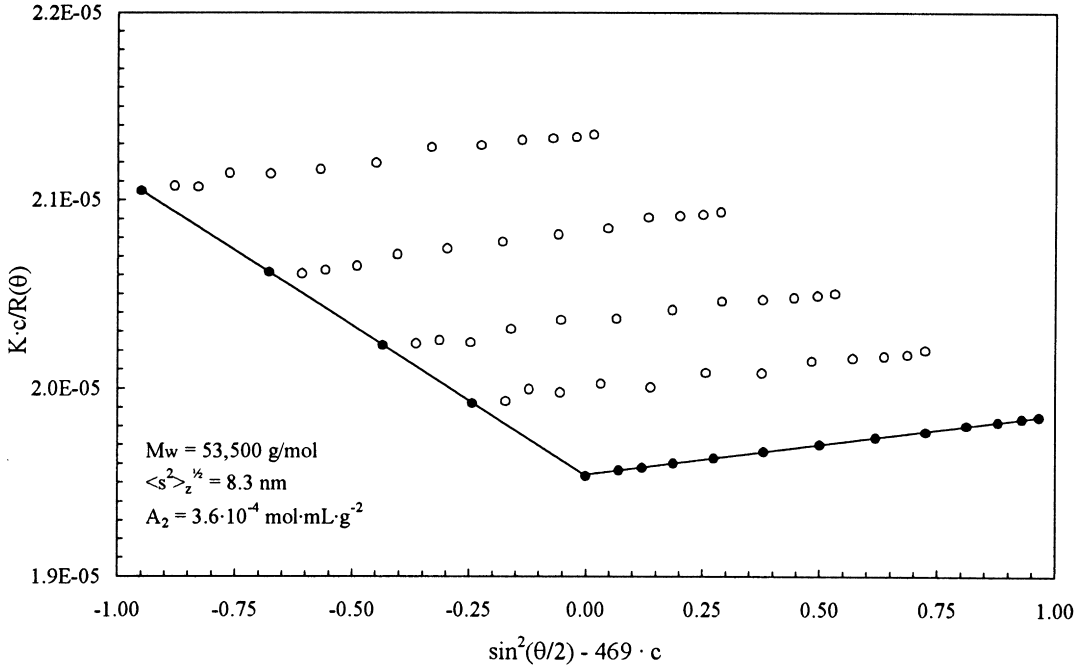

Fig. 3. Zimm plot of the $PHEA_1$ sample in the mobile phase at 25°C.

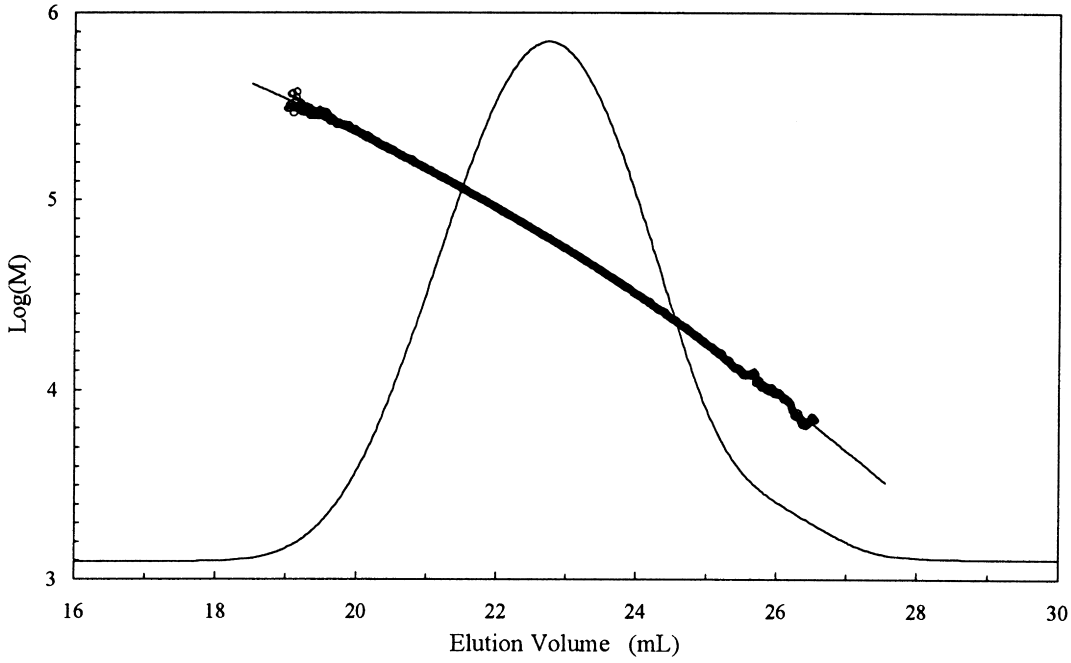

Fig. 4. Experimental calibration $M = f(V)$ of the SEC system by SEC–MALS.

detectors show good signal-to-noise ratio and the elution was substantially regular. The concentration of the PHEA sample was approximately 3 mg/ml. Despite their similar polymeric backbone, PHEA and PAHy show quite different molecular properties; in fact a good fractionation of PAHy requires a more complex basic mobile phase at pH ≅ 8. However, for comparison of the molecular properties of PHEA and PAHy, we have used the same mobile phase, 0.2 M NaCl + 0.1 M Tris pH 8, for both the polymers.

A summary of the results for the four PHEA samples is reported in Table 1. Table 1 summarizes the results obtained by three methods. The first method was the classical off-line batch mode MALS. The second method was the SEC on-line mode by the dual detectors MALS and DRI. The third method was the SCV viscometer on-line to the SEC system.

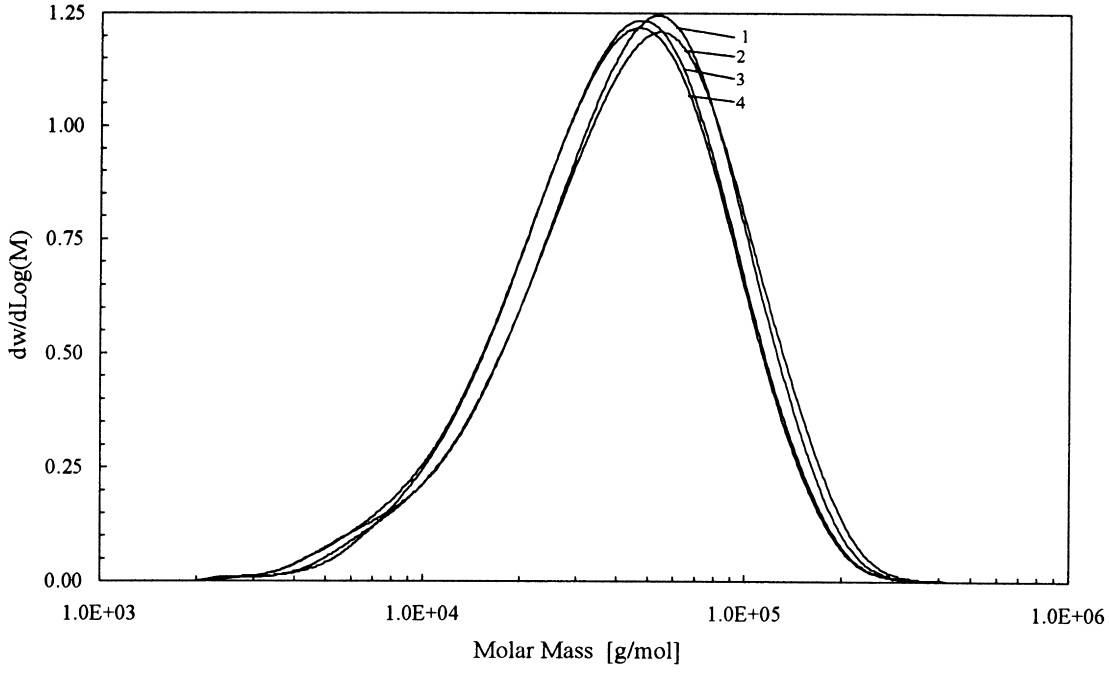

Fig. 5. Comparison of the differential molar mass distributions of four PHEA batches.

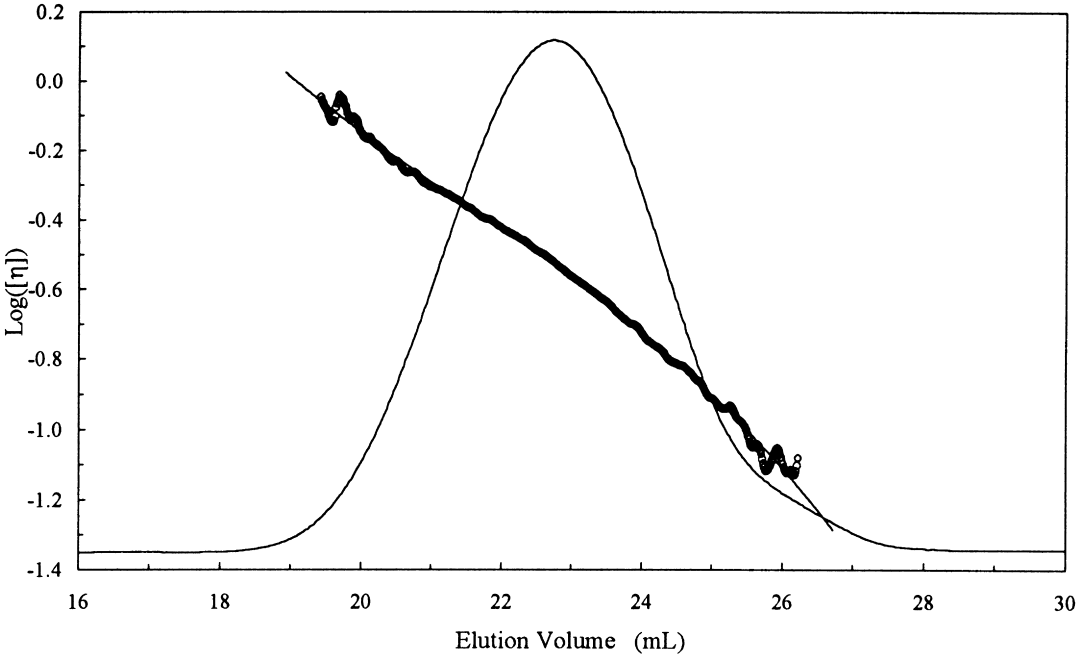

Fig. 6. Experimental calibration $[\eta] = f(V)$ of the SEC system by SEC–SCV.

Fig. 3 shows the classical elaboration, Zimm-plot, of a MALS off-line experiment with the $PHEA_1$ sample. The weight-average molar mass M_w of the sample was 53,500 g/mol, the second virial coefficient A_2 was 3.6×10^{-4} mol ml g^{-2} and the gyration radius R_g was 8.3 nm. The large positive value of the second virial coefficient proves that the used mobile phase was a good solvent for the PHEA polymer, unlike PAHy macromolecules for which in the same solvent, the second virial coefficient A_2 for PAHy, was negative. With regard to the size of the macromolecules the precision of the off-line MALS measure is relatively low because 8.3 nm lies in the lower measurable limit of the technique. However, using the on-line SEC–MALS method we have also obtained an estimation of the dimension of the PHEA macromolecules.

Fig. 4 shows the experimental molar mass calibration $M = f(V)$, where V denotes the elution volume of the SEC system by the on-line MALS detector with the $PHEA_1$ sample. Considering the high d*n*/d*c* value, 0.169 ml/g, and the relatively high molar mass of the PHEA samples compared with the PAHy samples, the signal-to-noise ratio was very good. A comparison of the differential MMD, by SEC–MALS, of the four PHEA samples is reported in Fig. 5. The MMDs of the four PHEA samples were fairly similar. As can be seen in Table 1, the M_w average of the four PHEA samples ranged from 46 to 53 K g/mol and the maximum M_w difference between the four samples was approximately 13%. Table 1 also shows the good agreement between the off-line M_w result 53.5 K g/mol and the on-line M_w result 53.0 K g/mol for the $PHEA_1$ sample.

The dispersity index D for PHEA was in general a little lower than 2. D substantially ranged from 1.7 to 1.9. It is well known that the MALS detector tends to underestimate the D value considering the low sensitivity to the low molar mass fractions. However the D values obtained from the SEC–SCV method and from the conventional SEC method (data not reported) were lower than 2 for all the four PHEA samples. Hence, the D values reported in Table 1, from SEC–MALS, were a good estimation of the true values.

The on-line SCV viscometer, coupled with the concentration detector, measures the intrinsic viscosity $[\eta]$ of each fraction. Fig. 6 shows the experimental calibration $[\eta] = f(V)$ of the SEC system by SEC–SCV obtained with the $PHEA_1$ sample. The measure of $[\eta]$ is not critical as the measure of the dimension of the macromolecules. As can be seen in Fig. 6 it is possible to estimate $[\eta]$ with good accuracy also for low molar mass fractions. The intrinsic viscosity of the whole sample $[\eta]$ has been estimated by the SCV data. $[\eta]$ for the four PHEA samples, see Table 1, ranged from 0.22 to 0.26 dl/g. Further, the SCV data has been used to estimate the $[\eta] = f(M)$ power law: MHS plot. To this goal, the on-line method presents some advantages. First, assuming ideal SEC fractionation, i.e. absence of band broadening, we have a quasi-uniform composition with respect to the molar mass of each slice of the chromatogram. Hence, for each slice, fraction, we can assume that $M_z \cong M_w \cong M_n$. Second, in the SEC–MALS–SCV characterization, after the fractionation on some SEC columns, we can analyse only the fractions of macromolecules with higher molar mass. Third, increasing the concentration of the sample to obtain better signal-to-noise ratio, we could extend the measurable limit towards lower values. Finally, with regard to the previous PAHy samples, we have to

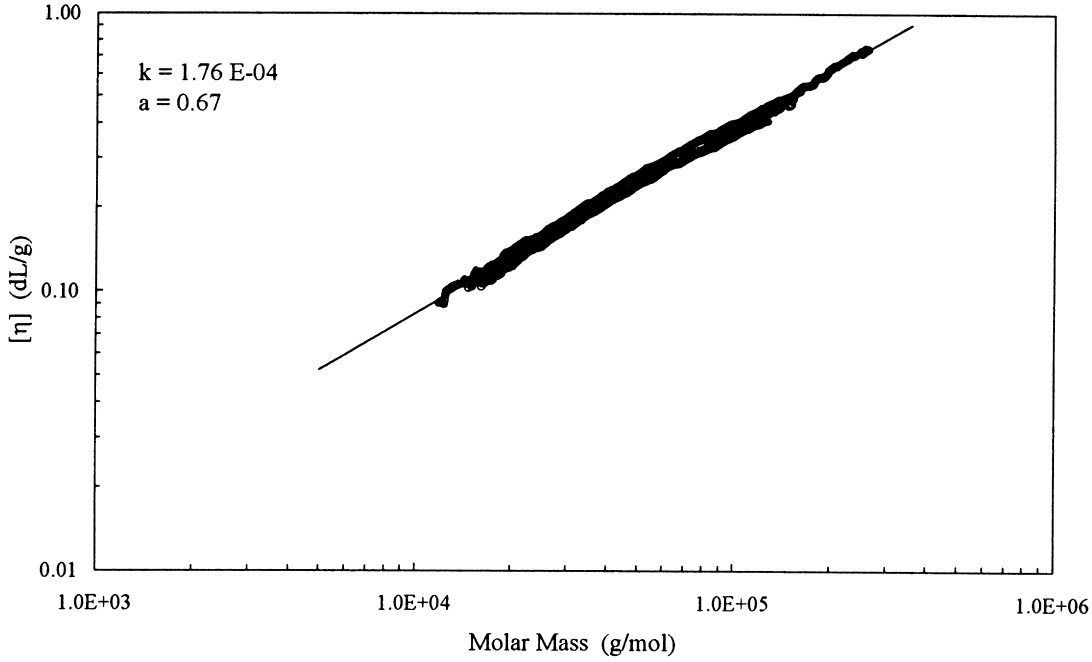

Fig. 7. $[\eta] = f(V)$ power law, MHS plot, for the PHEA polymer.

consider that the molar mass of the PHEA samples was substantially higher.

Each SEC–MALS–SCV chromatogram produces two direct experimental functions: $M = f(V)$ and $[\eta] = f(V)$. From these two experimental functions one obtains a third derived function: the $[\eta] = f(M)$ power law. Using this derived function, we have estimated the coefficients, intercept (k) and slope (a), of the MHS plot. To increase the accuracy of the measure we have used the following procedure. We have gathered the "good data region" of the four PHEA chromatograms. By good data region, we mean the region of the chromatogram where the signal-to-noise ratio was optimal. This procedure leads to a derivation of the power law coefficients from many experimental points and a wide range of molar mass. In the specific case we have used about 1200 points where M approximately ranged from

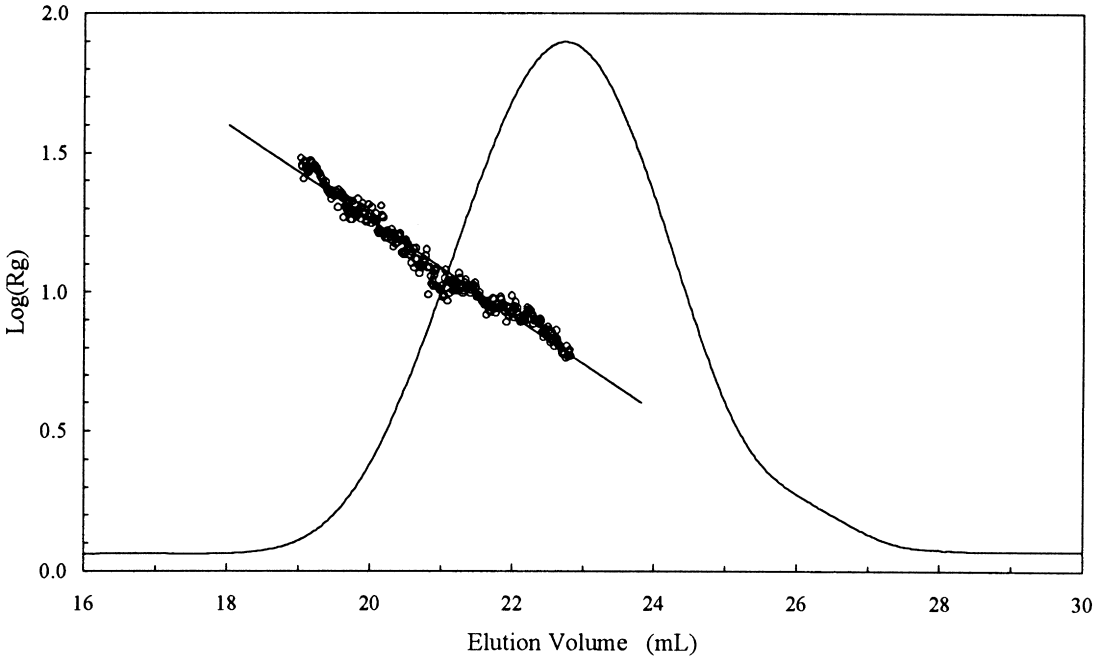

Fig. 8. Experimental calibration $R_g = f(V)$ of the SEC system by SEC–MALS.

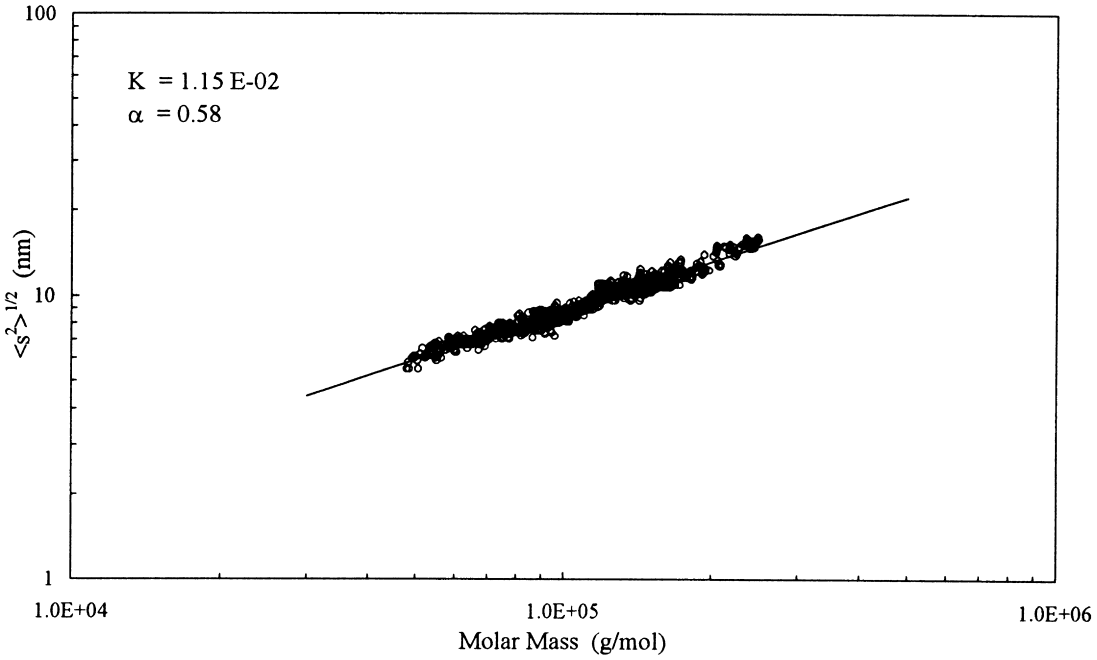

Fig. 9. $R_g = f(M)$ power law for the PHEA polymer.

1.0×10^4 to 2.5×10^5 g/mol. In this way the accuracy of the estimation of the coefficients increases considerably. Fig. 7 shows the $[\eta] = f(M)$ power law for PHEA was obtained with this procedure. The coefficients of the equation were: $k = 1.76 \times 10^{-4}$, $[\eta]$ is expressed in dl/g, and $a = 0.67$. The slope of the MHS plot for PHEA in the SEC mobile phase at 25°C is typical of random coil molecules in a good solvent. This result, considering the accuracy of the measure of M and $[\eta]$ by means of the on-line SEC–MALS–SCV system, is meaningful. Besides, the slope of the MHS plot found for PHEA is substantially higher than the slope found for PAHy, $a = 0.53$, in the same experimental conditions [11].

We have also tried to estimate the dimension of the PHEA macromolecules by the on-line SEC–MALS method. Measurement of the gyration radius by MALS requires that the angular dependence be experimentally measurable. R_g

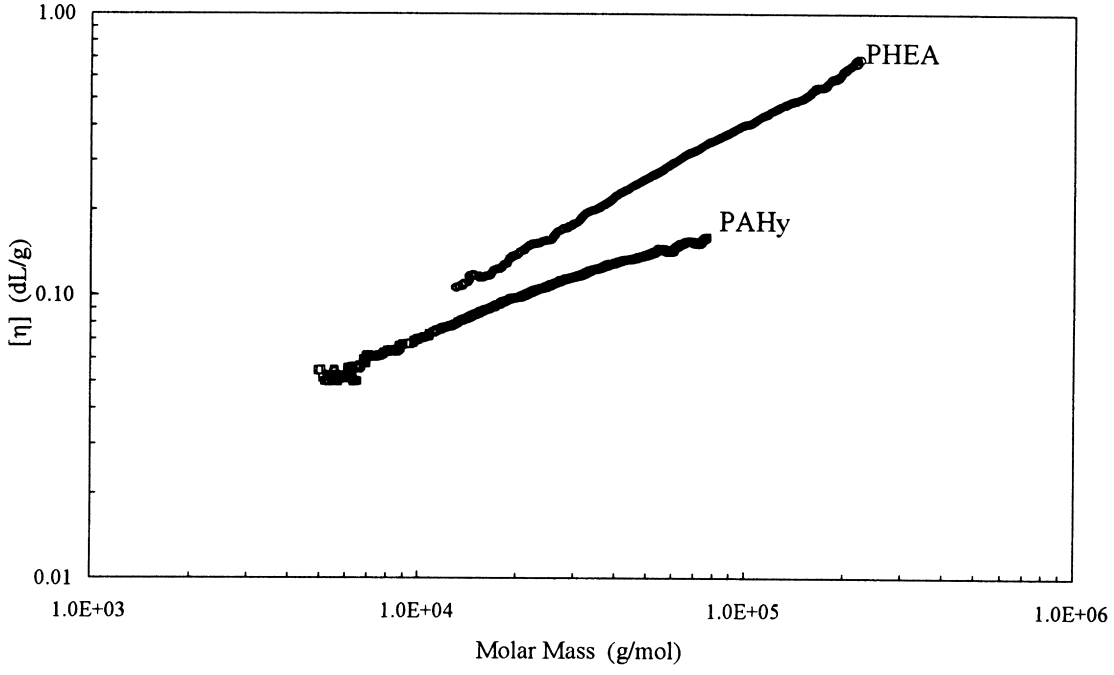

Fig. 10. Comparison of the MHS plot for PHEA and PAHy polymers.

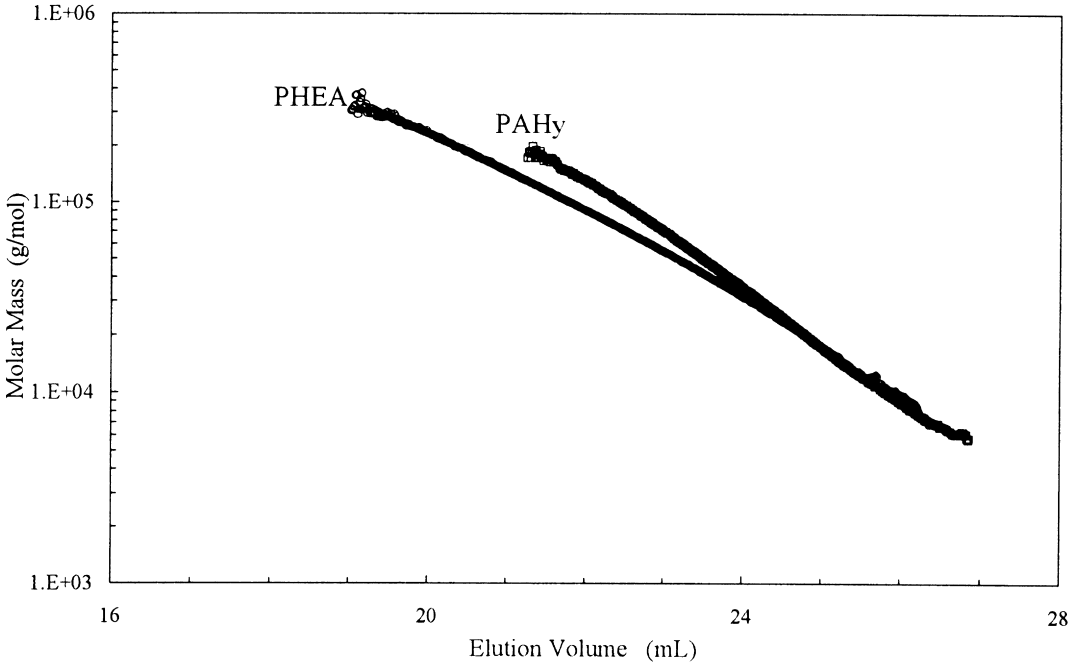

Fig. 11. Comparison of the $M = f(V)$ experimental calibration by SEC–MALS for PHEA and PAHy polymers.

resulting from unfractionated PHEA samples, Zimm plot, were substantially scattered. Hence we are oriented to use the on-line SEC–MALS method to estimate the size of the PHEA macromolecules. There are a lot of experimental results that confirm that the minimum measurable R_g with a He–Ne laser by an on-line SEC–MALS method on a well fractionated sample, is approximately 10–12 nm [23]. Obviously in the low range of values, the precision of the measure dramatically decreases. Fig. 8 shows the experimental calibration $R_g = f(V)$ by SEC–MALS. We can see the relatively wide range of molar mass in which the dimension of the PHEA macromolecules was measurable with acceptable accuracy. Using these data, from the four PHEA samples, we have estimated the coefficients, intercept (K) and slope (α), of the $R_g = f(M)$ power law. For this goal, we have used a procedure similar to that used for the MHS plot. That is, we have gathered the good data region of the four PHEA chromatograms. Fig. 9 shows the $R_g = f(M)$ power law for PHEA as constructed with the method described above. The coefficients of the equation were: $K = 1.15 \times 10^{-2}$ and $\alpha = 0.58$. Despite the relative precision of the measure, the slope of the $R_g = f(M)$ power law found for PHEA confirms the random coil conformation of the molecules. Besides, the slope of the $R_g = f(M)$ power law is in substantial agreement with the slope of the MHS plot.

The conformational analysis performed by means of the $[\eta] = f(M)$ and $R_g = f(M)$ power laws confirms that the PHEA macromolecules in aqueous solution are flexible coils. Further, considering the A_2 value, we can assert that the used mobile phase was a good solvent for PHEA. These results are in good agreement with the results obtained by the SAXS characterization for PHEA. Both MALS and SAXS results agree on the flexible conformation of the PHEA polymer, while results of the SAXS characterization suggested a more rigid conformation for the PAHy chain compared to the PHEA chain. The same conclusion was not drawn in the present study. Fig. 10 shows the comparison of the $[\eta] = f(M)$ power laws for PHEA and PAHy by the SEC–SCV system. We can see that, at constant molar mass, $[\eta]$ for PAHy was sensibly lower than $[\eta]$ for PHEA in the whole range of molar mass. Further, Fig. 11 shows the comparison of the $M = f(V)$ experimental calibration for PHEA and PAHy from SEC–MALS. Supposing ideal SEC fractionation, at constant hydrodynamic volume, the PAHy macromolecules show higher molar mass than the PHEA macromolecules. Therefore, both the MALS and SCV results are congruent with a more compact structure of the PAHy macromolecules compared to the PHEA macromolecules.

Often it is very useful to estimate the MMD of the polymer by the conventional SEC and universal calibration (SEC–UC) method [24]. The SEC–UC method is very attractive because it is relatively simple: the SEC–UC method uses only a single concentration detector. Hence, we have also estimated the MMD of the PHEA samples by the SEC–UC method. The universal calibration of the SEC system was constructed using some PEO and PEG narrow MMD standards. The coefficients of the MHS plot, in the mobile phase, were the following: $k = 2.894 \times 10^{-4}$ and $a = 0.702$ for PEO and $k = 1.117 \times 10^{-3}$ and $a = 0.572$ for PEG. Surprisingly, despite the absence of aggregation and of meaningful interaction of the macromolecules with the packing of the SEC columns the agreement between SEC–UC and SEC–MALS results was not good.

On average, on the four PHEA samples, the M_w value by SEC–UC was approximately 18% higher than the M_w value by SEC–MALS.

4. Conclusion

From the results obtained in the molecular characterization of PHEA, by means of a SEC–MALS–SCV chromatographic system, the following conclusions may be drawn. The fractionation of PHEA, on some Ultrahydrogel SEC columns, was relatively simple. Using an aqueous mobile phase of medium ionic strength, the elution was substantially regular and the macromolecules were not aggregate. The molar mass of the PHEA samples approximately ranges from 46 to 53 K g/mol and the intrinsic viscosity ranges from 0.22 to 0.26 dl/g. Both the slopes of the $[\eta] = f(M)$ and of the $R_g = f(M)$ power laws demonstrate that the PHEA macromolecules in aqueous solution are flexible coils. Besides, the second virial coefficient value proves that the used mobile phase was a good solvent for PHEA. These conformational results for PHEA are substantially different from the conformational results for PAHy. Reassuming, between PHEA and PAHy we can evidence some substantial differences: (i) the used SEC mobile phase is a good solvent for PHEA and a poor solvent for PAHy; (ii) PAHy conformation is more compact than the PHEA conformation; (iii) PHEA macromolecules, contrary to PAHy macromolecules, do not aggregate and do not interact with the packing of the SEC columns. Despite that, the estimation of the PHEA molar mass by the simple SEC–UC method was approximately 18% higher than that obtained with SEC–MALS system.

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