Available online at www.sciencedirect.com
SCIENCE DIRECT®
Polymer 44 (2003) 4871–4879
polymer
www.elsevier.com/locate/polymer

Molecular characterization of α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide derivatives as potential self-assembling copolymers forming polymeric micelles

R. Mendichi[a], A. Giacometti Schieroni[a], G. Cavallaro[b,*], M. Licciardi[b], G. Giammona[b]

[a]*Istituto per lo Studio delle Macromolecole (CNR), Via Bassini 15, 20133 Milano, Italy*
[b]*Dipartimento di Chimica e Tecnologie Farmaceutiche, Università degli Studi di Palermo, Via Archirafi 32, 90123 Palermo, Italy*

Received 13 January 2003; received in revised form 30 April 2003; accepted 30 May 2003

Abstract

A family of graft copolymers derivatives obtained from α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) have been studied as potential self-assembling macromolecules forming stable polymeric micelles at low critical micellar concentration. These polymers are obtained grafting on PHEA poly(ethylene glycol) (PEG) (M_w 5000 g/mol) (PHEA–PEG), hexadecylamine (PHEA–C_{16}) or both moieties (PHEA–PEG–C_{16}). The PHEA derivatives were characterised by a multi-angle light scattering (MALS) photometer on line to a size exclusion chromatography system in obtaining the molar mass distribution of the polymers. In addition, to investigate the capacity to form micellar aggregates in aqueous medium the MALS photometer was used in off-line batch mode in obtaining molar mass and dimension of the polymeric aggregates.
© 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Polyaspartamides; Micelles; Light scattering

1. Introduction

Micellisation of biologically active substances is a method commonly used to increase the solubility and biovailability of lipophilic drugs and nutrients [1–3]. The use of micelles as drug carriers can also: to prolong drug permanence time in blood circulation long enough to provide accumulation in the required area, to permit it to accumulate in body region with leaky vasculature, to target drug to action site by the attachment of a specific ligand to the outer micellar surface [1–3]. In addition micelles can be prepared in large quantities reproducible way and at low cost. Moreover, the drug can be effectively protected from eventual inactivation reaction in the biological surrounding and its toxic potential can be strongly reduced [1–3].

Currently used low-molar mass pharmaceutical surfactants have high solubilisation power towards poorly soluble pharmaceuticals but, in same cases, also high critical micellar concentration (CMC) and low stability upon strong dilution. On the other hand, amphiphilic copolymers are also known to form micellar systems in solution provided with very high solubilisation capacity and rather low CMC value that makes them very stable in vivo [4–6].

Polymeric micelles belong to a particular class of micelles; they are formed from copolymers consisting of both hydrophilic and hydrophobic portions that can be organised into a polymeric chain in different fashions forming random, block or graft copolymers [6].

To date, a large variety of copolymers may be used to build polymeric micelles [5,6] differing in polymeric backbone and in kind and amount of hydrophilic and hydrophobic residues, constituting polymeric surfactant molecules. Besides it reasonable to admit that to structural differences in amphiphilic copolymer, correspond a quite different physico-chemical properties of resulting aggregates such as CMC and micellar size. In particular, micelles formed by randomly modified grafted copolymers are usually smaller in size than micelles formed by end-modified block copolymers, since they can be formed within one polymeric chain providing micelles with a smaller aggregation number [7].

* Corresponding author. Tel.: +39-91-6236131; fax: +39-91-6177333.
E-mail address: gennacav@unipa.it (G. Cavallaro).

0032-3861/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0032-3861(03)00486-5

A family of polymers derived from α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) [8] have been studied in our laboratories as potential self-assembling macromolecules forming stable polymeric micelles at low CMC. PHEA is a synthetic polymer with a protein-like structure obtained by a simple reaction of ethanolamine with polysuccinimide (PSI), which is easily prepared by thermal polycondensation of D,L-aspartic acid [8,9]. PHEA presents ideal properties for drug delivery because it is highly soluble in water, non-toxic, non-antigenic, non-immunogenic [9]; its favourable toxicological properties allowed for proposing it as a plasma expander [9], drug carrier in the synthesis of macromolecular prodrugs [10–13] and as starting material after proper and easy derivatisation with glycidylmethacrilate to obtain UV or γ-irradiation crosslinkable biomaterials [14,15].

Moreover, an exhaustive and consistent molecular characterisation of PHEA has been obtained by the proper combination of techniques such as multi-angle light scattering (MALS), viscometry and size exclusion chromatography (SEC) and small angle X-rays (SAXS) [16,17].

In a previous paper we reported the synthesis and the biopharmaceutical characterisation of new PHEA copolymers obtained grafting on PHEA poly(ethylene glycol) (PEG) (M_w 5000 g/mol) (PHEA–PEG), hexadecylamine residues (PHEA–C_{16}) or both moieties (PHEA–PEG–C_{16}) [18]. Interestingly these studies demonstrated that the co-substitution of the PEG and the hydrophobic hexadecylalkyl residues yielded macromolecules whose biodistribution profile strongly suggested their arrangement into supramolecular structures [18].

Now, in the present paper we report an exhaustive molecular characterisation of PHEA–PEG, PHEA–C_{16} and PHEA–PEG–C_{16} by using MALS photometer both in off-line batch mode and on-line to a SEC system. The molar mass distribution (MMD) and the extent of aggregation of these PHEA copolymers have been also determined.

2. Experimental

2.1. Materials

O-(2-Aminoethyl)-*O′*-methylpolyethylene glycol 5000 (PEG–NH_2) (<0.17 mmol NH_2/g), hexadecylamine (C_{16}–NH_2) and all the other chemicals were furnished by Fluka (Buchs, Switzerland). Polystyrene (PS) narrow standard (M_w = 10,900 g/mol) was furnished from Polymer Standard Service (Mainz, D). Bovine serum albumin (BSA) was furnished from Sigma (Milan, I). Dimethylacetamide (DMAc) was furnished from Aldrich (Milan, I). Water solvent was MilliQ grade Millipore (Bedford, MA, USA). All other chemicals were of analytical grade.

PHEA was prepared by complete aminolysis of a PSI in dimethylformamide (DMF) solution keeping reaction temperature between 22 and 26 °C [8]. The PHEA derivatives containing PEG residues (PHEA–PEG) and both PEG and hexadecylamine groups (PHEA–PEG–C_{16}) were prepared and purified according to a previously reported procedure [18].

Briefly for the preparation of PSI–PEG copolymer a solution of PEG–NH_2 in DMF was added drop-wise to a solution of PSI in DMF and the mixture was kept for 30 h at room temperature.

For the preparation of PSI–PEG–C_{16} copolymer a solution of C_{16} in DMF was added drop-wise at 60 °C to a solution of PSI–PEG in DMF and the mixture reacted for 7 h at the same temperature.

For PHEA–PEG and PHEA–PEG–C_{16} copolymers proper amounts of ethanolamine were added drop-wise to DMF solutions of PSI–PEG or PSI–PEG–C_{16} copolymers maintaining the reaction under stirring for 3 h and keeping mixture temperature between 22 and 26 °C. All copolymers were purified by exhaustive dialysis running distilled water using Visking Dialysis tubing with a molecular cut off of 12,000–14,000.

Analytical and spectral data were in agreement with previously reported results [18].

The synthesis of PHEA–C_{16} copolymer was performed by using the following method that involves the preparation of PSI–C_{16} copolymer and the subsequent aminolysis of this derivative with ethanolamine.

For the synthesis of PSI–C_{16} copolymer a solution of C_{16}–NH_2 in DMF (0.75 g, 3.1 mmol/5 ml) was added drop-wise at 60 °C to a solution of PSI in DMF (0.4 g, 4.1 mmol of PSI repeating units/5 ml). The reaction mixture was maintained at 60 °C for 7 h under continuous stirring in the argon atmosphere, afterwards it was precipitated into ethyl ether, washed several times with the same solvent and finally dried under vacuum for several hours.

To a DMF solution of PSI–C_{16} (1 g/8 ml) 1.52 ml (2.5×10^{-2} mol) of ethanolamine were added drop-wise maintaining the reaction temperature between 22 and 26 °C under stirring for 3 h. The resulting polymer was precipitated into ethyl ether, washed several times with acetone to neutrality and finally dried under vacuum. The polymer was dispersed in water and purified by exhaustive dialysis running distilled water using Visking Dialysis Tubing with a molecular cut off 12,000–14,000. The solution was lyophilised and the purified product, obtained with a 95% yield (as based on starting PSI), were characterised by IR spectrophotometry and ^{1}H NMR analysis. The spectral data were in agreement with attributed structures.

IR spectra of PHEA–C_{16} (KBr) showed bands at: 3312 cm^{-1} (OH; –NH–), 2985 and 2854 cm^{-1} (C–H stretching of C_{16} chains), 1657 cm^{-1} (amide I), 1542 cm^{-1} (amide II) belonging to PHEA.

^{1}H NMR of PHEA–C_{16} (D_2O): δ 0.87 (t, 3H, –CH_2–CH_3), 1.28 (m, 28H, –CH_2–CH_2–CH_2–), 2.79 (m, 2H, –CO–CH–CH_2–CO–NH–), 3.34 (m, 2H, –NH–CH_2–CH_2–OH), 3.64 (m, 2H, –NH–CH_2–CH_2–OH), 4.72 (m, 1H, –NH–CH(CO)CH_2).

3. Methods

3.1. Instrumentation

This study was performed by a MALS Dawn DSP-F photometer from Wyatt (Santa Barbara, CA, USA) used both on-line to a SEC system and in off-line batch mode. The multi-detector SEC chromatographic system consisted of three on-line detectors: a MALS, a home-made single capillary viscometer (SCV), and a 410 differential refractometer (DRI) from Waters (Milford, MA, USA) as concentration detector. The on-line SCV detector was used in measuring the intrinsic viscosity (η) of the samples. This multi-detector SEC system has been described in detail previously [19,20]. Two SEC methods were used. First one consisted of 0.15 M NaCl as mobile phase, 35 °C, 0.8 ml/min and two Ultrahydrogel columns from Waters (1000 and 250 Å of pores size). Second one, consisted of DMAc as mobile phase, 80 °C, 0.6 ml/min and two mixed Jordigel GBR columns from Jordi (Bellingham, MA, USA). Also in the off-line batch mode a K5 flow cell was preferred to reduce the scattering volume. In batch mode for aggregation studies three different solvents at room temperature were used: 0.1 M phosphate buffer pH 7.4, 0.15 M NaCl pH 5.9, and 0.2 M NaCl + 0.1 M Tris pH 8. Each solution used in batch mode was prepared by mixing a weighted amount of the polymer with the solvent. Half of each solution was filtered through a 0.2 μm cellulose acetate filter, the other half one was filtered through a 0.45 μm filter.

The MALS photometer uses a vertically polarised He–Ne laser ($\lambda = 632.8$ nm) and simultaneously measures the intensity of the scattered light at 15 angular locations ranging in aqueous solvent from 14.5 to 158.3°. The calibration constant was calculated using toluene as standard assuming a Rayleigh factor of $1.406 \times 10^{-5}\ \mathrm{cm}^{-1}$. The normalisation of the photodiodes was performed by measuring the scattering intensity of a BSA globular protein in the aqueous solvent and of a narrow MMD PS standard ($M = 10,900$ g/mol) in DMAc solvent assumed to act as isotropic scatterers. Details of the MALS photometer have been described elsewhere [20].

The specific refractive index increment, dn/dc, of the polymers with respect to the solvents was measured by a Chromatix KMX-16 differential refractometer from LDC Milton Roy (Riviera Beach, FL, USA).

4. Results and discussion

Three polyaspartamide derivatives were synthesised by two subsequent aminolysis reactions of a polisuccinimide (PSI) with PEG–NH_2 and/or C_{16}–NH_2 in the first step and with ethanolamine in the second step [18]. The Degree of Derivatization% (DD%) for all copolymers (PHEA–PEG, PHEA–C_{16} and PHEA–PEG–C_{16}) was determined by ^{1}H NMR comparing the integral of a peak attributable to linked derivatising groups and the integral of a peak assigned to starting polymeric backbone by the following ratio: DD = (polyethylenglycol groups or hexadecyl groups/polymer repeating unit) × 100 mol; in particular for PHEA–PEG copolymer the DD% was calculated by comparing of the integral of the peak corresponding to protons at δ 3.69 assigned to –(O–CH_2–CH_2)– that belong to linked PEG with the integral of the peak related to protons at δ 2.82 assigned to –NH–CH(CO)–CH_2– belonging to PHEA; for PHEA–C_{16} copolymer the DD% was calculated by comparing of the integral of the peak corresponding to protons at δ 0.87 assigned to –CH_2–CH_3 (or the integral of the peak related to protons at δ 1.28 assigned to –CH_2–CH_2–CH_2–) that belong to linked C_{16} with the integral of the peak related to protons at δ 2.82 assigned to –NH–CH(CO)–CH_2– belonging to PHEA; both these methods were used to evaluate the DD% of PEG and C_{16} of the PHEA–PEG–C16 copolymer. The chemical structure of PHEA–PEG–C_{16} and PHEA–C_{16} are reported in Scheme 1.

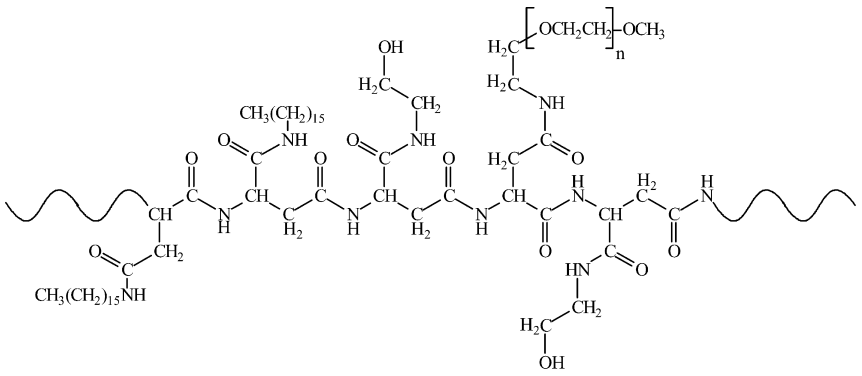
The content of PEG in PHEA–PEG and of C_{16} in PHEA–C_{16} were found equal to 1.8 and 8 mol%, respectively as well as the content of PEG and C_{16} in PHEA–PEG–C_{16} copolymer were found equal to 2.5 and 7.7 mol%, respectively. Each determination was the average of five evaluations on the same batch.

The grafting of hydrophilic and hydrophobic moieties like PEG and hexadecylamine, respectively on the PHEA backbone, suggested the possibility to promote, in aqueous phase, the polymer arrangement into micelle like supramolecular structures. In aqueous medium, we suppose that the PEG chains could be exposed to the external aqueous phase while the hexadecylamine residues could constitute an inner hydrophobic core; such a supramolecular system could act as colloidal vector for drug delivery of hydrophobic molecules solubilising them into the micellar nucleus.

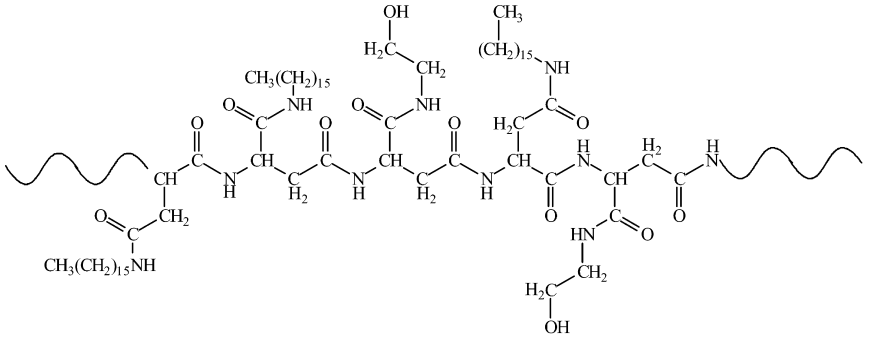
In order to investigate the molecular properties of these PHEA derivatives and their capacity to form large aggregates in aqueous medium the MALS photometer was used in obtaining the MMD of the polymers and the dimension of the polymeric aggregates [21]. Static light scattering (LS), also known as elastic LS or total intensity LS, is a convenient method in studying the molar mass and the size of macromolecules or particles in solution (aggregates, micelles, etc.). Following Zimm [22] in a LS experiment the reciprocal of the reduced Rayleigh factor ($R(\theta)$) may be expressed by the following equation:

$$\frac{Kc}{R(\theta)} = \frac{1}{M_w P(\theta)} + 2A_2 c \qquad (1)$$

where $R(\theta)$ denotes the excess of Rayleigh factor of the solution with regard to the pure solvent, $K = (2\pi^2 n_0^2 (\mathrm{d}n/\mathrm{d}c)^2)/(\lambda_0^4 N_a)$ is the optical constant, n_0 is the refractive index of the solvent, dn/dc is the refractive index increment of the solute with respect to the solvent, λ_0 is the wavelength of the light in the vacuum, N_a is the Avogadro's number, c is



PHEA-PEG-C16



PHEA-C16

Scheme 1.

the concentration of the solution, M_w is the weight-average molar mass, A_2 is the second virial coefficient and $P(\theta)$ is the intramolecular scattering function or 'form factor'. The dn/dc values for the PHEA homopolymer and three derivatives in 0.15 M NaCl and in DMAC solvents are reported in Table 1.

The scattering intensity of large molecules depends on the angle θ in consequence of the destructive interference. Using the MALS detector the angular variation of the scattering is directly measured by an array of photodiodes located at 18 different angles. In such a way $P(\theta)$ is obtained directly at each instant. $P(\theta)$ is defined as the ratio between $R(\theta)$ in presence of interference ($\theta > 0°$) and $R(\theta)$ in absence of interference ($\theta = 0°$). Usually $R(\theta)$ at different angles is measured and $R(\theta = 0°)$ is calculated by an extrapolation to zero angle. Debye [23] showed that the form factor $P(\theta) = R(\theta)/R(\theta = 0°)$ may be approximated by:

$$P(\theta) = 1 - \frac{1}{3}\mu^2 Rg^2 + \ldots \quad (2)$$

where $\mu = (4\pi/\lambda)\sin(\theta/2)$ is a function of the angle θ and of the wavelength λ of the light in the medium. As a result from a MALS off-line batch experiment at some concentrations three important parameter (M_w, A_2 and Rg) could be obtained by a double extrapolation to infinite dilution ($c = 0$) and zero angle ($\theta = 0$). In a SEC–MALS run the extrapolation to infinite dilution is not possible because at

Table 1
dn/dc values for the PHEA homopolymer and derivatives in 0.15 M NaCl and DMAc solvents

Polymer	0.15 M NaCl	DMAc
PHEA	0.169	0.220
PHEA–PEG	0.154	0.190
PHEA–C_{16}	0.170	0.215
PHEA–PEG–C_{16}	0.160	0.191

each elution volume only a single concentration is known. Consequently using the MALS detector on-line to a SEC system the term $2A_2c$ of the Eq. (1) is neglected and only M_w and Rg could be obtained.

The MMD of PHEA homopolymer obtained in 0.15 M NaCl mobile phase was reported previously [16]. In the present study the MMD of the PHEA derivatives in a different solvent (DMAc) able to solubilise to molecular level both the PHEA homopolymer and the three derivatives was investigated using the MALS photometer on-line to a SEC system. However, like for the PHEA homopolymer the SEC fractionation of the PHEA–PEG derivative does not present particular problems. SEC fractionation of the PHEA–PEG derivative can be performed using 0.15 M NaCl solvent as mobile phase. Fig. 1 shows the normalised DRI signal of the PHEA and of the PHEA–PEG derivative and the comparison of experimental $\log(M) = f(V)$ experimental functions, where V is the elution volume, of the two samples from the on-line MALS detector.

It is well known that the light scattering detector is particularly sensible to the presence of aggregates. Typically in presence of the aggregates the MALS signal presents at low elution volumes a pre-peak and/or shoulders. Fig. 1 clearly shows the absence of aggregates in the PHEA–PEG solution. Evidently the grafting of a hydrophilic moiety like as PEG on the PHEA chain does not change the behaviour of the macromolecules in aqueous solution. Table 2 summarises more important data, M_w and dispersity index D, of the characterisation of PHEA and PHEA–PEG derivative in aqueous solvent. PHEA data were very similar to those presented in our previous study [16,18].

The molar mass of the PHEA–PEG derivative is lower with respect to the starting PHEA: respectively $M_w = 49,100$ for PHEA and $M_w = 33,100$ for PHEA–PEG. This result was well expected since the preparation of PHEA–PEG involves two subsequent reactions with primary amines (PEG–NH_2 and ethanolamine) on PSI macromolecules and the reaction of primary amines with PSI

Table 2
Summary of the SEC–MALS characterisation of the PHEA homopolymer and PHEA–PEG, PHEA–PEG–C_{16} and PHEA–C_{16} derivatives

Polymer	0.15 M NaCl		DMAc		
	M_w (g/mol)	D	M_w (g/mol)	D	(η) (dl/g)
PHEA	49,100	1.7	48,900	1.8	0.26
PHEA–PEG	33,100	1.6	34,400	1.6	0.22
PHEA–C_{16}			20,600	1.8	0.11
PHEA–PEG–C_{16}			36,600	1.9	0.13

determine not only the aminolysis of succinimide rings but also a partial hydrolysis of PSI chains.

The SEC fractionation of the PHEA derivatives containing a hydrophobic moiety like as hexadecylamine resulted be more and more complex. Such PHEA derivatives, namely PHEA–C_{16} and PHEA–PEG–C_{16}, in aqueous solvent under the same experimental conditions used for PHEA, were strongly aggregate and the molar mass recovered from the on-line light scattering detector was overestimated. In addition, the chromatograms of these derivatives were abnormal with evident shoulders and long tails. In order to evaluate the true MMD of these PHEA–C_{16} and PHEA–PEG–C_{16} derivatives, we had to use a different SEC method consisting of DMAc as organic mobile phase and two adequate organic SEC columns (see Section 3 for details). Fig. 2 shows the normalised DRI signal, in DMAc mobile phase, of PHEA and of its three copolymers.

In such a plot we can see the absence of shoulders and tails in the chromatogram of both PHEA and all the derivatives. The comparison of the raw signals of the MALS detector (90° photodiode) and of the DRI detector of a derivative containing the hydrophobic C_{16} moiety, namely PHEA–C_{16}, is reported in Fig. 3.

Also the LS signal of the PHEA–C_{16} derivative, as well as of the PHEA–PEG–C_{16} derivative (not shown), is deprived of pre-peak and shoulders. This fact confirms that these PHEA derivatives in DMAc solvent were not aggregate and the MMD could be estimated with a good

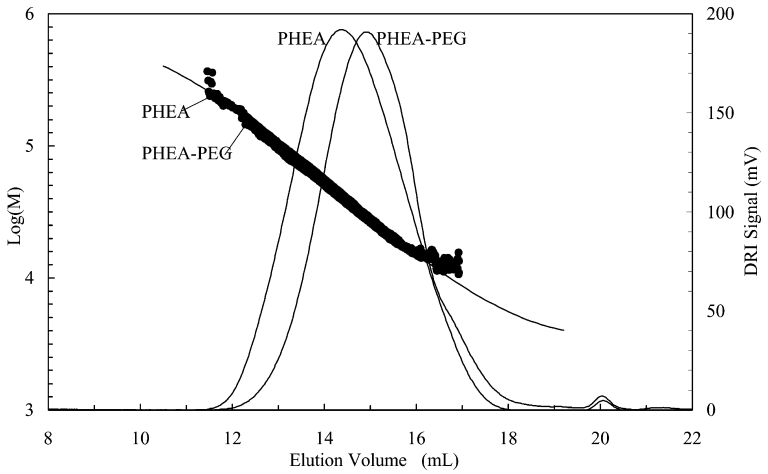


Fig. 1. Normalised DRI signal of PHEA and of PHEA–PEG derivative in aqueous mobile phase and the relative $\log(M) = f(V)$ experimental functions.

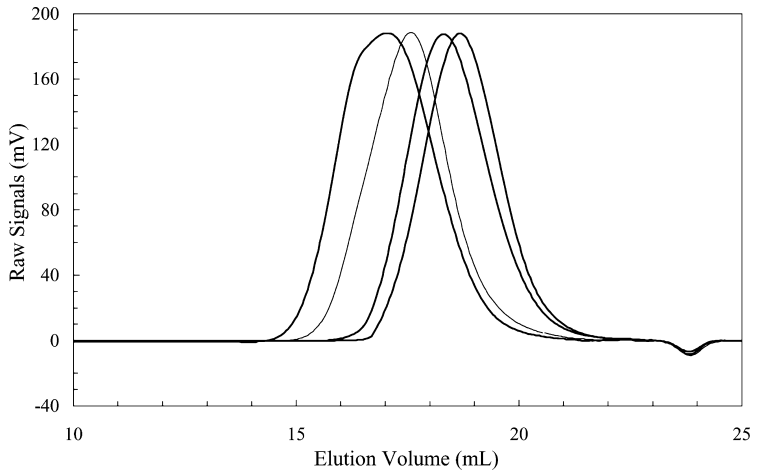


Fig. 2. Comparison of the normalized DRI signal in DMAc mobile phase of PHEA and of three derivatives. Respectively, from left to right: PHEA, PHEA–PEG, PHEA–C_{16} and PHEA–PEG–C_{16}.

approximation. Really M_w was 20,600 g/mol for PHEA–C_{16} and 36,600 g/mol for PHEA–PEG–C_{16}; respectively D was 1.8 and 1.9. In addition the recovered M_w and D data obtained for PHEA and PHEA–PEG in DMAc were very similar to that obtained in aqueous solvent.

PHEA derivatives are copolymers. As it is well known since the chemical composition of a copolymer could be not constant, the molar mass recovered from an LS photometer could be only apparent. On the other hand, the difference between apparent and true molar mass depends on the extent of the non-homogeneity in chemical composition of considered copolymers. However, it is worthy to note that M_w and (η) results, see Table 2, are consistent both for PHEA and derivatives and it is well known that (η) data from the on-line viscometer does not depend on the chemical composition of the polymer. Hence we can conclude that at least in first approximation M_w and D results reported in Table 2 were quite close to the 'true' values.

The MALS photometer used in off-line batch mode is a convenient method in studying the aggregation of macromolecules in solution [24]. We were interested to estimate molar mass, size and average aggregation number of the PHEA derivatives in aqueous medium. In the specific case the molar mass of the non-aggregate polymer (defined as nominal value) was known (Table 2). Thus the weight-average number of macromolecules in a micelle (N_{Aggr}) was estimated directly by the ratio between the apparent molar mass of the aggregate (M_w) and the nominal molar mass value (M_n).

Fig. 4 shows the comparison of the scattering intensity of PHEA and of the three derivatives.

The MALS signals were obtained at nominal constant concentration (0.1 mg/ml) (CAC, critical concentration aggregation determined by surface tension measurements in different aqueous media, equal to an average of 0.04 mg/ml for PHEA–PEG–C_{16} and 0.08 mg/ml PHEA–C_{16}—paper in preparation) and with polymeric solutions filtered by 0.45 μm filters. As we can see the scattering intensity of the two derivatives, PHEA–C_{16} and PHEA–PEG–C_{16}, with respect to PHEA and PHEA–PEG, is impressive. In addition, Fig. 4 shows for the PHEA–C_{16}

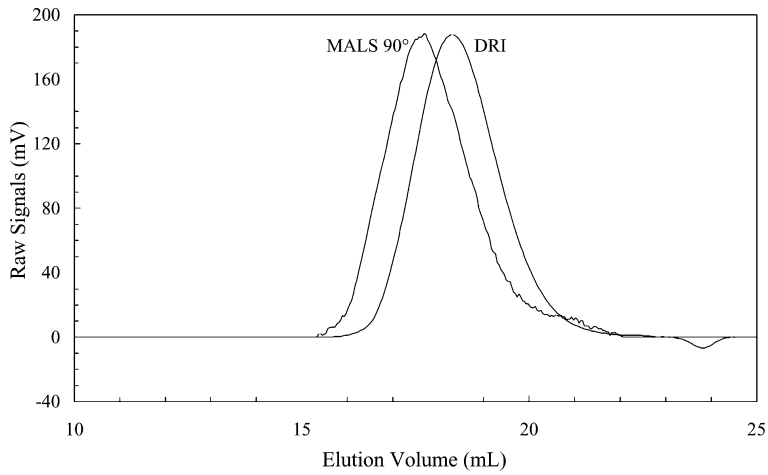


Fig. 3. Raw signals of the MALS detector (90° photodiode) and of the DRI detector of the PHEA–C_{16} derivative in DMAc mobile phase.

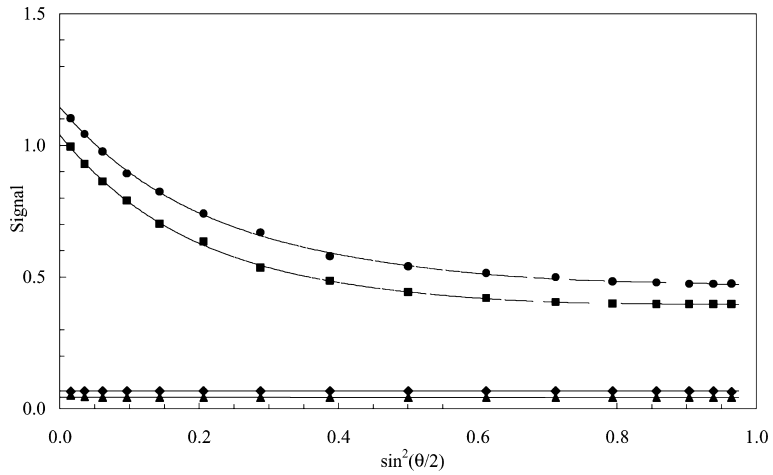


Fig. 4. Comparison of the scattering intensity in aqueous medium, 0.1 M phosphate buffer pH 7.4 and 0.2 μm filter, of PHEA (◆), PHEA–PEG (▲), PHEA–C_{16} (■) and PHEA–PEG–C_{16} (●).

and PHEA–PEG–C_{16} derivatives a meaningful angular variation of the scattering intensity that means large dimensions of the aggregates. On the contrary, PHEA and PHEA–PEG solutions do not evidence any presence of aggregates. This last consideration was coherent with the previous finding obtained using the on-line SEC–MALS system. Therefore, at least from a qualitative point of view, we found that PHEA–C_{16} and PHEA–PEG–C_{16} derivatives were able to form micelles in aqueous medium.

The MALS technique also permits a quantitative estimation of the molar mass and of the size of the aggregates. In the limit of very low concentration and scattering angle the molar mass and in particular the size of the macromolecules could be obtained with a good approximation also using a single concentration [24]. Hence, considering the extremely diluted solutions used in this study we have not performed the extrapolation to infinite dilution.

The weight-average molar mass (M_w) and the gyration radius (Rg) of the two derivatives PHEA–C_{16} and PHEA–PEG–C_{16} were determined both at different pH medium (pH 5.9, 7.4 and 8) and ionic strength and after filtering solutions by 0.2 and by 0.45 μm filters; the results of these experiments are summarised in Table 3.

As it can be seen, the results obtained after filtering solutions by 0.45 μm filter were quite dissimilar both at different pH and ionic strength and they were not congruent among them. On the other hand, by a quantitative evalution of the difference between the amount of copolymers (either PHEA–C_{16} and PHEA–PEG–C_{16}) that were retained on 0.45 and 0.2 μm filters the determination of the amount of polymeric aggregates characterised by high molecular weight was found to be negligible (about 1% w/w); for this reason we considered just data obtained though filtration on 0.2 μm filter; in this contest we can see that M_w decreases with increasing pH values for PHEA–C_{16} copolymers micellar systems, while the same trend was not found for PHEA–PEG–C_{16} copolymer that showed N_{aggr} always lower than that obtained with PHEA–C_{16}.

Finally, as an example Fig. 5 shows the $P(\theta)^{-1}$ vs. $\sin^2(\theta/2)$ plot of PHEA and of PHEA–C_{16} and PHEA–PEG–C_{16} derivatives using 0.1 M phosphate buffer pH 7.4 as solvent. The gyration radius Rg of the micelles estimated from the angular variation of the scattering was 14.9 nm for the PHEA–C_{16} derivative and 17.5 nm for the PHEA–PEG–C_{16} derivative.

Table 3
Summary of PHEA–C_{16} and PHEA–PEG–C_{16} derivatives aggregation data by off-line MALS in 0.15 M NaCl pH 5.9, 0.1 M phosphate buffer pH 7.4 and 0.2 M NaCl + 0.1 M Tris pH 8 at room temperature

Polymer	Solvent	Filter 0.20 μm			Filter 0.45 μm		
		M_w (Kg/mol)	Rg (nm)	N_{Aggr}	M_w (Kg/mol)	Rg (nm)	N_{Aggr}
PHEA–C_{16}	0.15 M NaCl, pH 5.9	228.3	22.7	20	509.1	45.6	45
PHEA–C_{16}	0.1 M PBS, pH 7.4	198.6	14.9	18	570.0	74.6	50
PHEA–C_{16}	0.2 M NaCl + 0.1 M Tris pH 8	162.4	16.9	14	194.0	18.2	16
PHEA–PEG–C_{16}	0.15 M NaCl, pH 5.9	180.0	30.8	10	226.5	44.3	11
PHEA–PEG–C_{16}	0.1 M PBS pH 7.4	216.2	17.5	11	626.0	84.0	32
PHEA–PEG–C_{16}	0.2 M NaCl + 0.1 M Tris pH 8	139.5	10.9	8	165.6	18.7	10

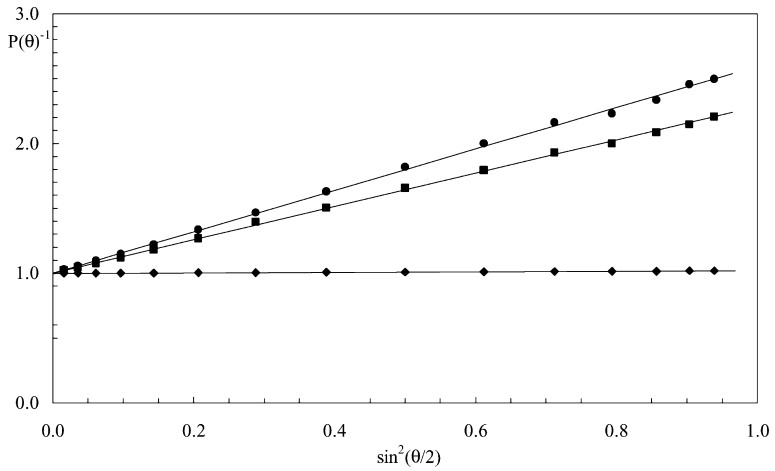


Fig. 5. $P(\theta)^{-1}$ *Vs.*$\sin^2(\theta/2)$ plot in 0.1 M phosphate buffer pH 7.4 solvent and 0.2 μm filter of PHEA (◆), PHEA–C_{16} (■) and PHEA–PEG–C_{16} (●).

5. Conclusions

In the present study the MMD of PHEA and some PHEA copolymers was investigated using the MALS photometer on-line to a SEC system and the extent of aggregation of these copolymers in aqueous medium was investigated using the MALS photometer in off-line batch mode. In particular we demonstrated that PHEA derivatives containing a hydrophobic moiety like hexadecylamine, namely PHEA–C_{16} and PHEA–PEG–C_{16}, in aqueous solvent were strongly aggregated and different SEC methods consisting in organic and aqueous mobile phases and two adequate SEC columns were used in order to evaluate the true MMD of these PHEA derivatives. In the organic mobile phase, in particular PHEA derivatives were not aggregate and the MMD could be estimated with a good approximation resulting equal to 20,600 and 36,600 g/mol for PHEA–C_{16} and PHEA–PEG–C_{16}, respectively. In addition, the MALS photometer in off-line batch mode was used to study the aggregation of C_{16} copolymers in aqueous solvent, at different pH values and ionic force. Obtained results evidenced that M_w of PHEA–C_{16} copolymer micellar systems decreases increasing pH values, while the same trend was not found for PHEA–PEG–C_{16} copolymer that showed N_{aggr} always lower than that obtained with PHEA–C_{16}. This results was explained in terms of improvement of self-assembling capability given from PEG to C_{16} copolymers that reduces the aggregation number of PEG containing copolymers.

Acknowledgements

We thank Italian CNR and MIUR for financial support.

References

[1] Muranishi S. Absorption enhancers. Crit Rev Ther Drug Carrier Syst 2000;7:1–33.
[2] Corrigan OI, Healy AM. In: Swarbrick J, Boylan JC, editors. Encyclopedia of pharmaceutical technology, vol. 3. New York: Marcel Dekker; 2002. p. 2639–53.
[3] Lasic D. Mixed micelles in drug delivery. Nature 1992;335:279–80.
[4] Kwon GS, Okano T. Polymeric micelles as new drug carriers. Adv Drug Deliv Rev 1996;21:107–16.
[5] Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv Drug Deliv Rev 2001;47:113–31.
[6] Torchilin VP. Structure and design of polymeric surfactant-based drug delivery systems. J Controlled Release 2001;73:137–72.
[7] Winnik FM, Davidson AR, Hamer GK, Kitano H. Amphiphilic poly(*N*-iopropylacrylamide) prepared by using a lipophilic radical initiator: synthesis and solution properties in water. Macromolecules 1992;25:1876–80.
[8] Giammona G, Carlisi G, Palazzo S. Reaction of α,β-poly(*N*-hidroxyethyl) -DL-aspartamide with derivatives of carboxylic acids. J Polym Sci Polym Chem 1987;25:2813–8.
[9] Neri P, Antoni G, Benvenuti F, Cocola F, Gazzei G. Synthesis of α,β-poly(*N*-hydroxyethyl)-DL-aspartamide, a new plasma expander. J Med Chem 1973;16:893–7.
[10] Giammona G, Carlisi B, Pitarresi G, Fontana G. Hydrophilic and hydrophobic polymeric derivatives of anti-inflammatory agents such as alclofenac, ketoprofen and ibuprofen. J Bioact Compat Polym 1991;6:129–41.
[11] Giammona G, Puglisi G, Cavallaro G, Spadaro A, Pitarresi G. Chemical stability and bioavailability of acyclovir coupled to α,β-poly(*N*-hydroxyethyl)-DL-aspartamide. J Controlled Release 1995;33:261–71.
[12] Giammona G, Cavallaro G, Pitarresi G, Fontana G, Carlisi B. Coupling of the antiviral agent zidovudine to polyaspartamide and in vitro drug release studies. J Controlled Release 1998;54:321–31.
[13] Cavallaro G, Pitarresi G, Licciardi M, Giammona G. Polymeric prodrug for release of an antitumor agent by specific enzymes. Bioconjugate Chem 2001;12:143–51.
[14] Giammona G, Tomarchio V, Pitarresi G, Cavallaro G. Glycidyl methacrylate derivatization of α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide and α,β-polyasparthydrazide. Polymer 1997;38:3315–23.
[15] Giammona G, Pitarresi G, Cavallaro G, Buscemi S, Saiano F. New biodegradable hydrogels based on a photo crosslinkable modified

polyaspartamide: synthesis and characterization. Biochem Biophys Acta 1999;1428:29–38.

[16] Mendichi R, Giammona G, Cavallaro G, Giacometti Schieroni A. Molecular characterization of α,β-poly[(*N*-hydroxyethyl)-DL-aspartamide] by light scattering and viscometry studies. Polymer 2000;41:8649–57.

[17] Coviello T, Yuguchi Y, Kajiawara K, Giammona G, Cavallaro G, Alhaique F, Palleschi A. Conformational analysis of α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) and α,β-polyasparthydrazide (PAHy) polymers in aqueous solution. Polymer 1998;39:4159–64.

[18] Caliceti P, Quarta SM, Veronese FM, Cavallaro G, Pedone E, Giammona G. Synthesis and biopharmaceutical characterization of new poly(hydroxyethyl)aspartamide copolymers as drug carriers. Biochem Biophys Acta 2001;1528:177–86.

[19] Mendichi R, Giacometti Schieroni A. Use of the single-capillary viscometer detector on-line to a size exclusion chromatography system with a new pulse-free pump. In: Provder T, editor. ACS Symposium Series, vol. 731. Washington, DC: American Chemical Society; 1999. p. 66–83.

[20] Mendichi R, Giacometti Schieroni A. In: Pandalai SG, editor. Current trends in polymer science, vol. 6. Trivandrum, India: Trans-World Research Network; 2001. p. 17–32.

[21] Wyatt PJ. Light scattering and the absolute characterization of macromolecules. Anal Chim Acta 1993;272:1–40.

[22] Zimm BH. Apparatus and methods for measurement and interpretation of the angular variation of light scattering; preliminary results on polystyrene solutions. J Chem Phys 1948;16:1099–116.

[23] Debye P. Molecular-weight determination by light scattering. J Phys Colloid Chem 1947;51:18–31.

[24] Mele A, Mendichi R, Selva A. Non-covalent associations of cyclomaltooligosaccharides (cyclodextrins) with *trans*-β-carotene in water: evidence for the formation of large aggregates by light scattering and NMR spectroscopy. Carbohydr Res 1998;310:261–7.