Amphiphilic polyaspartamide copolymer-based micelles for rivastigmine delivery to neuronal cells

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C. Scialabba, F. Rocco, M. Licciardia, G. Pitarresia, M. Ceruti, G. Giammona

Abstract

A novel polysorbate-80 (PS_{80})-attached amphiphilic copolymer comprising a hydrophilic α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) backbone and hydrophobic squalenyl-C_{17} (Sq_{17}) portions was synthesized and characterized; the formation of polymeric micelles was also evaluated. Rivastigmine free-base (Riv), a hydrophobic drug employed to treat Alzheimer's disease, was chosen as model drug to investigate micelle's ability to incorporate hydrophobic molecules and target them to neuronal cells. Micelle formation was studied through analyses including fluorescence spectroscopy and 2D ^{1}H-NMR NOESY experiments. Finally, the capacity of Riv-loaded micelles, versus free drug, to penetrate mouse neuroblastoma cells (Neuro2a) was evaluated. 2D ^{1}H-NMR NOESY experiments demonstrated that the PHEA-EDA-Sq_{17}-PS_{80} copolymer self-assembles into micelle structures in water, with a micelle core formed by hydrophobic interaction between Sq_{17} alkyl chains. Fluorescence probe studies revealed the CAC of PHEA-EDA-Sq_{17}-PS_{80} micelles, which was 0.25 mg mL^{-1}. The micelles obtained had a nanometric hydrodynamic diameter with narrow size distribution and negative surface charge. The PHEA-EDA-Sq_{17}-PS_{80} micelles incorporated a large amount of Riv, and the system maintained the stability of Riv after incubation in human plasma. An *in vitro* biological assay evidenced no cytotoxic effects of either empty or loaded micelles on the neuronal cell lines tested. Moreover, the micelles are internalized by neuroblastoma cell lines with drug uptake depending on the micelles concentration.

Introduction

The selective delivery of drug molecules to specific tissues or cells is an objective that is increasingly close to being achieved, thanks to the development of nanotechnologies applied to the pharmaceutical field (Liu, 2011). However, numerous factors, some physiological, others relating to chemical and physical properties of the drug still present significant obstacles to therapeutic success. Drug delivery to specific areas of the body, such as for example the central nervous system (CNS) is particularly difficult. In this case, a further obstacle consists of the blood–brain barrier (BBB), which acts as a biological filter (Pardridge, 1999; Abbott et al., 2006). The BBB is a mechanical membrane that separates the blood from the brain. It consists of endothelial cells connected by tight junctions, and a pool of enzymes, receptor transporters and efflux pumps. The BBB's function is to protect the brain from various foreign substances, such as neurotoxins; it thus comprises an insurmountable barrier to most drugs, including antibiotics, cytostatics and other essential CNS-active drugs.

Possible approaches to achieving an efficient drug concentration in the brain includes high-dose therapy and invasive methods, but, in both cases, the therapeutic effect is accompanied by numerous harmful side effects. Noninvasive approaches thus remain a challenge, that has given rise to the development of new drug-targeting technologies. One of the possible ways of delivering drugs to the brain is to use nano-scaled drug-delivery systems, such as nanoparticles, liposomes, dendrimers, or polymeric micelles (Yang, 2010). Numerous mechanisms where nanoparticulate systems can cross the BBB and enter into brain to deliver drugs have been reported, including endocytosis (Lockman et al., 2004; Park, 2009), opening of the tight junctions between endothelial cells, retention of nanoparticulate systems in the brain blood capillaries, and fluidization of endothelial cell membrane lipids (surfactant effect) to enhance drug permeability through the BBB (Kreuter, 2001). In this connection, it has frequently been found that, when exposed on the nanoparticles's surface, amphiphilic substances, such as polysorbate-80 (PS_{80}), increase the nanoparticles's capability to cross the BBB through the endocytosis mechanism (Kreuter, 2002). This property of polysorbate has been attributed to the possible adsorption of apolipoprotein E and/or B from the blood on the polysorbate-coated nanoparticles; the nanoparticles could thus mimic low-density lipoproteins and interact with the lipoprotein receptors

located on the brain capillary endothelial cells, and subsequently cross the cell membrane via receptor-mediated endocytosis (Kreuter, 2001). This study addresses the delivery of a model CNS-active drug to neuronal cells, by means of polymeric micelles obtained by self-assembling PS_{80}-attached amphiphilic copolymers.

Among nanoparticulate systems, polymeric micelles have recently emerged as promising carriers for drug delivery and targeting. They maintain drug levels in the therapeutically desirable range and increase drug solubility, stability, permeability and half-life. Polymeric micelles can be obtained by self-assembling of amphiphilic copolymers in which, in aqueous solution, hydrophilic and hydrophobic portions form a stable core-shell structure (Voets et al., 2006;Carlsen and Lecommandoux, 2009); they are capable of delivering a variety of drugs, including hydrophobic drugs whose clinical application is limited by their low solubility in aqueous solutions. They also improve delivery efficiency and reduce side effects by means of targeted delivery (Know and Okano, 1996).

Specifically, we report the synthesis and characterization of a novel amphiphilic copolymer with a hydrophilic α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) backbone and hydrophobic portions in the side chain consisting of C_{17}-squalenyl molecules. PS_{80} was attached to the resulting amphiphilic copolymer as targeting agent for CNS. PHEA is a synthetic water-soluble, biocompatible, nontoxic and nonantigenic polymer, which has been used for the preparation of colloidal drug-delivery systems, such as nanoparticles (Licciardi et al., 2011), micelles (Civiale et al., 2009; Craparo et al., 2011), and hydrogels (Pitarresi et al., 2008), for controlled drug deliver and to prepare polyelectrolytic complexes for gene delivery (Cavallaro et al., 2008; Cavallaro et al., 2010).

Squalene is a linear triterpene and an extremely hydrophobic molecule. It is used for numerous vaccine and drug-delivery emulsions because it facilitates solubilization, modified release, and cell uptake of drugs, adjuvants and vaccines (Christopher, 2009). Squalene is a biocompatible substance, found in different foods, including olive oil, carrots, rice and lettuce, and is a precursor in the biosynthesis of cholesterol, in animals and humans (Smith, 2000). Moreover, squalene has been shown to posses various beneficial physiological properties, including anticancer and antioxidant activities (Kelly, 1999).

Micelle formation was demonstrated through analyses including fluorescence spectroscopy and 2D ^{1}H-NMR NOESY experiments. Rivastigmine free-base (Riv), a drug used in treating Alzheimer's disease, was chosen as model drug in order to investigate the ability of these micelles to incorporate hydrophobic molecules. Finally, the capacity of Riv-loaded micelles, versus free drug, to penetrate into mouse neuroblastoma cells (Neuro2a) was evaluated.

Materials and methods

Materials

All reagents used were of analytical grade, unless otherwise stated. α,β-Poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and purified by a procedure reported elsewhere (Giammona et al., 1987; Mendichi et al., 2000). D,L aspartic acid, anhydrous *N,N*-dimethylformamide (DMF), anhydrous *N,N*-dimethylacetamide (DMA), bis(4-nitro-phenyl)carbonate (PNPC), pyrene, deuterated dimethylsulfoxide (DMSO-d_6), deuterated water (D_2O) (isotopic purity 99.9%) and polysorbate-80 (PS_{80}) were purchased from Sigma-Aldrich (Italy). Anhydrous dimethylsulfoxide (DMSO), ethylenediamine (EDA) and diethyl ether were purchased from Fluka (Italy). Rivastigmine hydrogentartrate was donated from Novartis Pharma AG (Switzerland).

Synthesis of PHEA-EDA-Sq17-PS80 copolymer

PHEA-EDA was synthesized by a procedure reported elsewhere (Licciardi et al., 2006). Briefly, 83 mg mL^{-1} of PHEA were dissolved in anhydrous DMF. This solution was added drop-wise to a solution of PNPC in anhydrous DMF (120 mg mL^{-1}), and the mixture obtained was allowed to react for 1 h at 40°C. EDA was then added (1 mL) and the final solution was stirred for 4 h at 25°C. The reaction mixture was purified, and characterized as reported elsewhere (Licciardi et al., 2006). PHEA-EDA was obtained with a yield of 97%, based on the initial amount of PHEA. The degree of derivatization in EDA (DD_{EDA}), determined by ^{1}H-NMR in D_2O and calculated by a method reported elsewhere (Licciardi et al., 2006), was 30.0±0.5 mol%.

To synthesize PHEA-EDA-Sq_{17} copolymer, C_{17}-squalenyl aldeide (SqCHO-C_{17}) (10 mg mL^{-1}), whose synthesis is reported elsewhere (Ognibene et al., 2011), dissolved in acetic acid/anhydrous DMF (10 vol.%) was added to a PHEA-EDA solution (33 mg mL^{-1}) in anhydrous DMF and left to react at 25°C for 4 h.

The amount of SqCHO-C_{17} was added according to R = (moles of SqCHO-C_{17}/moles of amino pendant groups in PHEA-EDA) = 0.1.

The resulting copolymer was precipitated in ethyl ether, and the product was washed four times with the same solvent and then once with acetone. The reaction mixture was purified, and characterized as reported elsewhere (Ognibene et al., 2011). PHEA-EDA-Sq_{17} was recovered with a yield of 90% (wt.%).

The ^{1}H-NMR (DMSO-d_6) spectrum of PHEA-EDA-Sq_{17} shows peaks at δ 1.52-1.61 (m, 12H, squalenyl allylic CH_3), 1.94–2.35 (m, 12H, squalenyl allylic CH_2), 2.79 (m, 2H, –CH–C**H₂**–CO–NH–), 3.13 (m, 2H, –NH–CH_2–C**H₂**–NH_2), 3.24 (m, 2H, –NH–C**H₂**–CH_2–O–), (m, 2H, –NH–C**H₂**–CH_2–NH_2), 3.50 (t, 2H, –NH–CH_2–C**H₂**–O–), 3.97 (m, 2H, –NH–CH_2–C**H₂**–O(CO)NH–CH_2–CH_2–NH_2), 4.55 (m, 1H, –NH–C**H**(CO)CH_2–), 4.98–5.10 (m, 3H, squalenyl vinylic CH), 7.25 (m, 1H, –C**H**=N–).

The degree of derivatization in Sq_{17} (DD_{Sq}), expressed as the mean value of three determination, resulted to be 3±0.3 mol%.

PHEA-EDA-Sq_{17}-PS_{80} was synthetized by a procedure reported elsewhere (Craparo et al., 2008). Briefly, PS_{80} was activated with PNPC, 267 mg mL^{-1} of PS_{80} and 62 mg mL^{-1} of PNPC were dissolved in 0.8 mL of anhydrous DMA under argon. This mixture was left to react for 3 h at 10°C. After this activation time, a solution of PHEA-EDA-Sq_{17} in a mixture of anhydrous DMA/anhydrous DMSO (4/1), under argon, was added drop-wise, and the final solution was left to react for 3 h at 25°C and overnight at 20°C, under continuous stirring.

The amounts of PS_{80} and PNPC were added according to *X* = (moles of PNPC/moles of PS_{80}) = 1 and *Y* = (moles of PS_{80}/ moles of amino pendant groups in PHEA-EDA-Sq_{17}) = 1.

The reaction mixture was purified, and characterized as reported elsewhere (Craparo et al., 2008). PHEA-EDA-Sq_{17}-PS_{80} was obtained with a yield of 85 wt.% based on the initial amount of PHEA-EDA-Sq_{17}.

The ^{1}H-NMR (DMSO-d_6) spectrum of PHEA-EDA-Sq_{17}-PS_{80} shows peaks at δ 0.85 (m, 3H, –CH_2–C**H₃**), 1.24 (m, 22H, –CH_2–C**H₂**–), 1.52–1.61 (m, 12H, squalenyl allylic CH_3), 1.94–2.35 (m, 12H, squalenyl allylic CH_2), 2.79 (m, 2H, –CH–C**H₂**–CO–NH–), 3.13 (m, 2H, –NH–CH_2–C**H₂**–NH_2), 3.24 (m, 2H, –NH–C**H₂**–CH_2–O–), (m, 2H, –NH–C**H₂**–CH_2–NH_2), 3.50 (t, 2H, –NH–CH_2–C**H₂**–O–), (t, 80H, –CH_2–C**H₂**–O–) 3.97(m, 2H, –NH–CH_2–C**H₂**–O(CO)NH–CH_2–CH_2–NH_2), 4.55 (m, 1H, –NH–C**H**(CO)CH_2–), 4.98–5.10 (m, 3H, squalenyl vinylic CH), (m, 2H, –CH=CH– of PS_{80}), 7.11 (m, 1H, –C**H**=N–).

The degree of derivatization in PS_{80} (DD_{PS80}), expressed as the mean value of three determination was 1.2±0.03 mol%.

Preparation of micelles

Rivastigmine free-base (Riv) was obtained by treatment of a 20 mg mL^{-1} aqueous solution of rivastigmine hydrogentartrate with 1 N NaOH until pH = 9,0. After 30 min, the drug was extracted five times from the aqueous phase with 20 mL of CH_2Cl_2 and dried under vacuum. The product was analyzed by ^{1}H-NMR and UV spectroscopy. The analytical and spectral data were in agreement with the theoretical data.

Drug was loaded into the polymeric micelles by milling known amounts of drug (10 mg) and copolymer (40 mg) in the dry state and then dissolved in DMF, a solvent in which both drug and copolymer are soluble. The organic solvent was then removed under vacuum, and a solid residue was obtained. Progressive addition of aliquots of water (until 5 mL) lead to self-assembling of the copolymer into micelle aggregates, and subsequently to the incorporation of drug molecules in the hydrophobic core. The dispersion was then left overnight under continuous stirring and excess drug was removed from the system by filtration on 0.45 µm filters and the resulting dispersion was finally freeze-dried. The final yield was 85%. Riv loading capacity of micelles was determined by a HPLC method (Waters Breexe System Liquid Chromatograph equipped with a Waters 717 Plus Autosampler, and a Shimadzu UV-VIS HPLC detector on line with a computerized workstation.) using a reversed-phase column and 0.01 M sodium heptane sulfonate (SHS)/CH_3CN (70:30 v/v) as mobile phase at a constant flow rate of 1.0 mL min^{-1}(Rao et al., 2005). The eluate was monitored at wavelength 217 nm. The drug peak was quantitatively determined by comparison with a standard calibration curve. For the analysis, 5 mg of rivastigmine-loaded PHEA-EDA-Sq_{17}-PS_{80} micelles (Riv-loaded micelles) were dissolved in 1 mL of mobile phase and 50 µl of this solution was analysed by HPLC after filtration thought 0.2 µm RC filter.

Empty PHEA-EDA-Sq_{17}-PS_{80} micelles were prepared following the above described procedure but in absence of Riv.

Determination of critical aggregation concentration

The critical aggregation concentration (CAC) of PHEA-EDA-Sq_{17}-PS_{80} was determined by fluorescence analysis, using pyrene as probe. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorophotometer. A stock solution of pyrene (6.0×10^{-5} M in acetone) was prepared and then aliquots of 20 µL were placed into vials and evaporated to remove acetone in an orbital shaker at 37°C. Subsequently, 2 mL of aqueous copolymer solution at concentrations ranging from 1×10^{-5} to 1 mg mL^{-1} were added to the pyrene residue; the final concentration of pyrene was 6.0×10^{-7} M in each sample. The solutions were kept at 37°C for 24 h under continuous stirring to equilibrate pyrene with micelles. Pyrene excitation and emission spectra were recorded at 37°C using an emission wavelength of 373 nm and an excitation wavelength of 333 nm.

Dynamic light scattering analysis and ζ potential measurements

The mean diameter, width of distribution (polydispersity index, PDI) and ζ potential of the micelles were measured at 25°C using a Zetasizer NanoZS instrument, fitted with a 532 nm laser at a fixed scattering angle of 90°. The intensity-average hydrodynamic diameter (size in nm) and polydispersity index (PDI) of empty and Riv-loaded PHEA-EDA-Sq_{17}-PS_{80} micelles in double distilled water (0.2 mg mL^{-1}) were obtained by cumulate analysis of the correlation function. The ζ potential (mV) was calculated from the electrophoretic mobility using the Smoluchowsky relationship and assuming

that *Ka* >> 1 (where *K* and *a*are the Debye-Hückel parameter and particle radius, respectively). Each experiment was performed in triplicate.

Cytotoxicity assay on mouse neuroblastoma (Neuro2a) cells

The cytotoxicity of empty and Riv-loaded PHEA-EDA-Sq_{17}-PS_{80} micelles was evaluated on mouse neuroblastoma (Neuro2a) cell lines, purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna, Italy. The cytotoxicity studies were assessed by MTT assay. Briefly, cells were seeded in 96 well plate at a density of 5×10^4 cells/well and grown in Minimum Essential Medium (MEM) with 10% FBS (fetal bovine serum) and 1% of penicillin/streptomycin (10,000 U mL^{-1}penicillin and 10 mg mL^{-1} streptomycin) at 37°C in 5% CO_2 humidified atmosphere. After 72 h of incubation, cells were treated with Riv-loaded micelles solutions in bidistilled water, having micelle concentrations of 1, 0.5 and 0.25 mg mL^{-1}. Aliquots of micelle solutions (10 µL) were added to the cells in 100 µL fresh medium and incubated for 6, 24 and 48 h. After incubation, 20 µL of MTT reagent solution [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide, Sigma; 0.5 mg mL^{-1})] were added to each well and plates were incubated at 37°C for 2 h; the absorbance was then measured by a multiwell plate reader (Multiskan Ex, Thermo absystems, Finland), at 490 nm after background correction. Cells treated with Riv solutions in DMSO, with drug concentration of 0.1, 0.2 and 0.05 mg mL^{-1}, were used as positive control; untreated cells were used as negative control.

Cell drug uptake studies

To analyze drug and drug-loaded copolymer micelle uptake by neuronal cells, Neuro2a cell line ($2\cdot\times 10^5$ cells·mL^{-1}) maintained in normal medium were cultured in a 24-well plate at 37°C in an atmosphere of 5% CO_2 for 1–2 h until they formed a confluent monolayer. Upon reaching confluence, the culture medium was removed and cells were washed twice with 0.5 mL of Dulbecco's modified Eagle's medium (DPBS, Sigma, Italy) and then 0.5 mL of fresh MEM was added. After pre-incubation at 37°C for 30 min, aliquots of micelle-encapsulated drug or drug suspension were added to each well, to achieve final drug concentrations of 0.1, 0.2 and 0.05 mg mL^{-1}, then incubated for 6 h. Following the incubation period, the medium was removed and the cell monolayer was washed twice with DPBS and treated with an appropriate volume of CelLytic MT reagent (Sigma-Aldrich, Italy). After incubation for 15 min on a shaker, cells were scraped and the cell lysate collected and lyophilized. Drug uptake was quantified by analyzing cell lysate and washing media by HPLC, after redispersion in distilled water. It is noteworthy that the sum of internalized and non-internalized drug was in all cases close to 100% (data not reported).

Stability studies in human plasma

The *in vitro* stability of drug and of drug-loaded copolymer micelle were evaluated by incubating samples with human plasma at 37±0.1°C. Six suspensions, three containing 2 mg of Riv-loaded micelles in 10 mL of human plasma, and three containing free rivastigmine in human plasma at a concentration of 0.2 mg mL^{-1} were prepared and maintained at 37°C under mechanical stirring. At suitable time intervals (6, 24 and 48 h) 2 mL of 10% trifluoroacetic acid (v/v) were added to precipitate plasma proteins. After immediate mixing and centrifugation for 5 min at 10,000 rpm at 4°C supernatants were filtered through a 0.2 µm regenerated-cellulose (RC) membrane filter and analyzed by HPLC. All experiments were carried out in triplicate.

Statistical analysis

The statistical analysis of the samples was performed by using a Student's *t*-test and *p*-values <0.05 were considered statistically significant. All data were reported as means ± SD, unless otherwise stated.

Results and discussions

Synthesis and characterization of PHEA-EDA-Sq17-PS80 copolymer

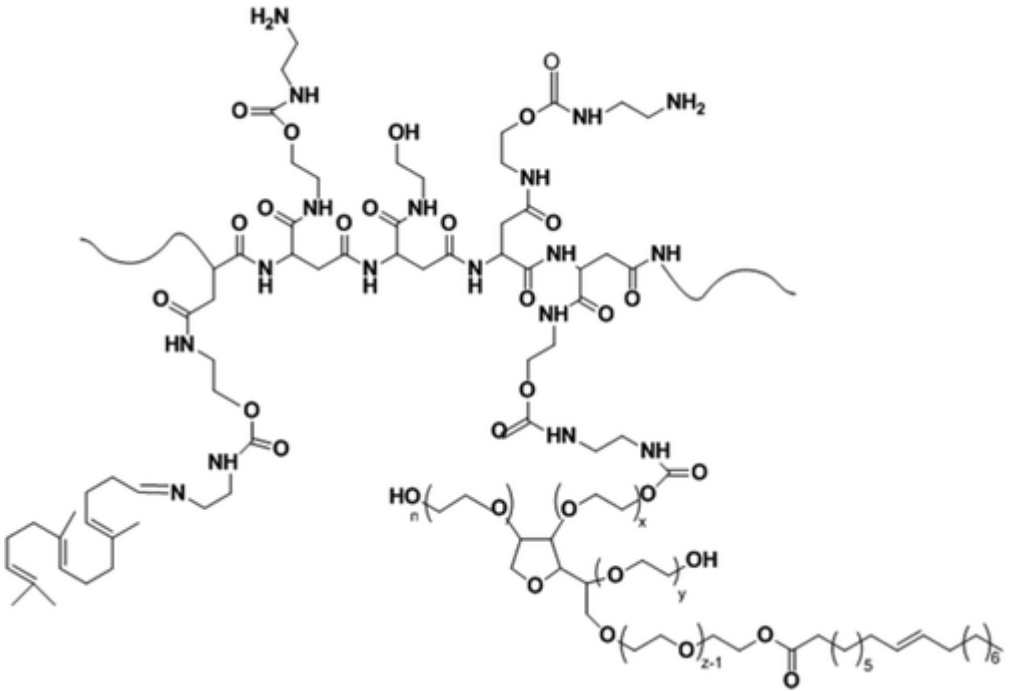
The synthesis of the studied amphiphilic copolymer, PHEA-EDA-Sq_{17}-PS_{80}, started from the polymer PHEA, a synthetic copolymer with a protein-like structure, and comprised three successive reactions. PHEA was first partially functionalized with EDA to introduce amine pendant moieties into the polymeric backbone, leading to PHEA-EDA (Licciardi et al., 2006), with a DD_{EDA} of 30.0±0.5 mol% related to PHEA monomer units. In this connection, we calculate that each PHEA-EDA copolymer macromolecule has about 60 amine pendant moieties in its backbone.

Then, in order to add the appropriate amount of hydrophobic chains to the hydrophilic copolymer, PHEA-EDA was derivatized with a squalene derivative, SqCHO-C_{17}. The aldehyde functional group of SqCHO-C_{17} easily reacted with the amine groups on the polymeric side-chain of PHEA-EDA, in the presence of an organic acid, forming the immine group. The DD_{Sq} of PHEA-EDA-Sq_{17} was calculated by the method reported elsewhere (Ognibene et al., 2011). In particular, it was determined by comparing the integral peaks related to protons at δ 1.52–1.61 and 1.94–2.35, attributable to squalenyl allylic CH_3 and squalenyl allylic CH_2 respectively with signals at δ 2.79 and 4.55 assigned to protons of the PHEA backbone. In this case we calculated that each PHEA-EDA copolymer macromolecule has about 6 Sq_{17} pendant portions in its backbone, corresponding to 20±1% (mol/mol) of EDA pendant groups (3.0±0.3 mol% referred to PHEA monomer units).

In the third step, the reaction between PHEA-EDA-Sq_{17} and PS_{80} was carried out by using PNPC as activating agent of PS_{80}alcoholic groups, and the subsequent reaction with the nucleophilic amine pendant groups of PHEA-EDA-Sq_{17}, led to the PHEA-EDA-Sq_{17}-PS_{80} amphiphilic copolymer. This surfactant was chosen for the well-known ability of polysorbate surface-coated nanoparticles to transport loaded drugs across BBB (Kreuter, 2001; Schroeder et al., 1998).

The PHEA-EDA-Sq_{17}-PS_{80} copolymer, properly purified, was characterized through ^{1}H-NMR spectroscopy, which confirmed the presence of PS_{80} chains on the PHEA backbone and enabled the molar derivatization degree (DD_{PS80}%) to be calculated. The DD % which indicates the percentage of linked polysorbate chains versus repeating units of PHEA, was calculated by comparing the integral of the peaks related to protons at δ 1.24 and protons at δ 3.50 assigned to –CH_2–C**H_2**– and CH_2–C**H_2**–O– belonging to linked PS_{80}, with the integral of the peaks related to protons at δ 2.79, attributable to –CH–C**H**$_2$–CO–NH– belonging to the PHEA backbone, as reported elsewhere (Craparo et al., 2008). The molar percent of PS_{80} linked to PHEA was 1.2±0.03% (mol/mol) versus polymer repeating units. As an example, the chemical structure of PHEA-EDA-Sq_{17}-PS_{80} is shown in Figure 1.

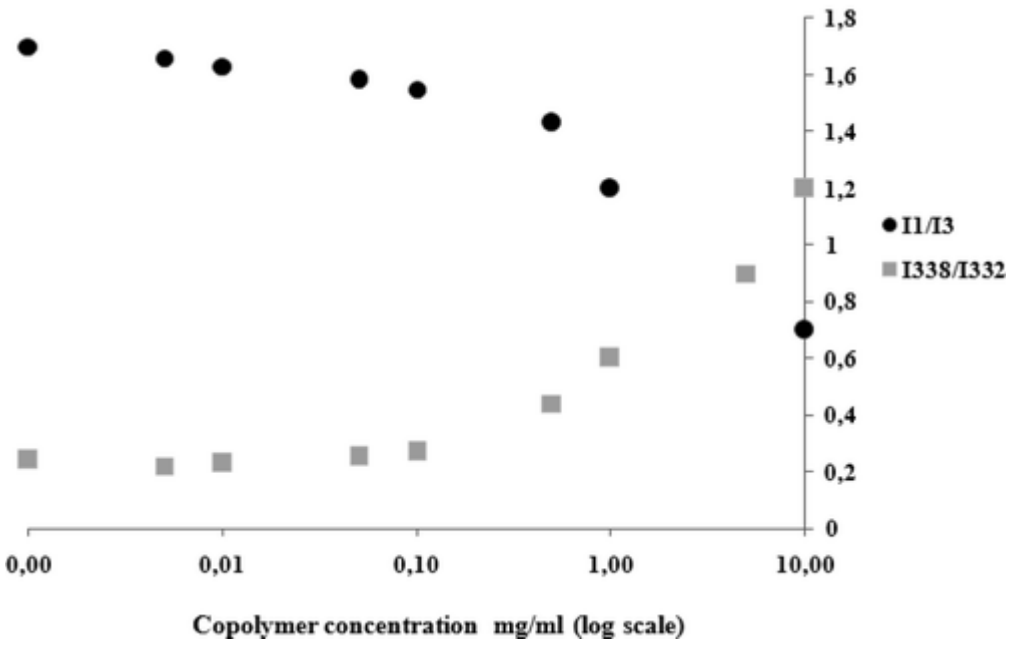
Figure 1. Chemical structure of PHEA-EDA-Sq_{17}-PS_{80} copolymer ($x + y + z + n = 2$).



Preparation and characterization of empty PHEA-EDA-Sq17-PS80 micelles

The simultaneous presence in the PHEA-EDA-Sq_{17}-PS_{80} copolymer of hydrophobic and hydrophilic portions makes this macromolecules suitable to form polymeric micelles by self-assembling in aqueous dispersion. Micelles formation in aqueous medium was investigated through fluorescence studies using pyrene as fluorescent probe. Pyrene, being a hydrophobic molecule, tends to segregate from aqueous solution and enter the inner hydrophobic core of micelles, thus changing its photo-physical properties. Indeed, the intensity ratio I_1/I_3 of pyrene changes from ≈1.8 in water to ≈0.6 in non-polar solvents such as hexane (Chen et al., 1999). Figure 2 shows the curves, which can be used for CAC determination, of the I_{338}/I_{332} ratio, obtained from the excitation spectra, and the I_1/I_3 ratio, obtained from pyrene emission spectra recorded at 37°C, against the logarithm of the copolymer concentrations.

Figure 2. I_{338}/I_{332} intensity ratio calculated from pyrene excitation spectra and I_1/I_3 intensity ratio calculated from pyrene emission spectra in water, as a function of the logarithm of copolymer concentration.

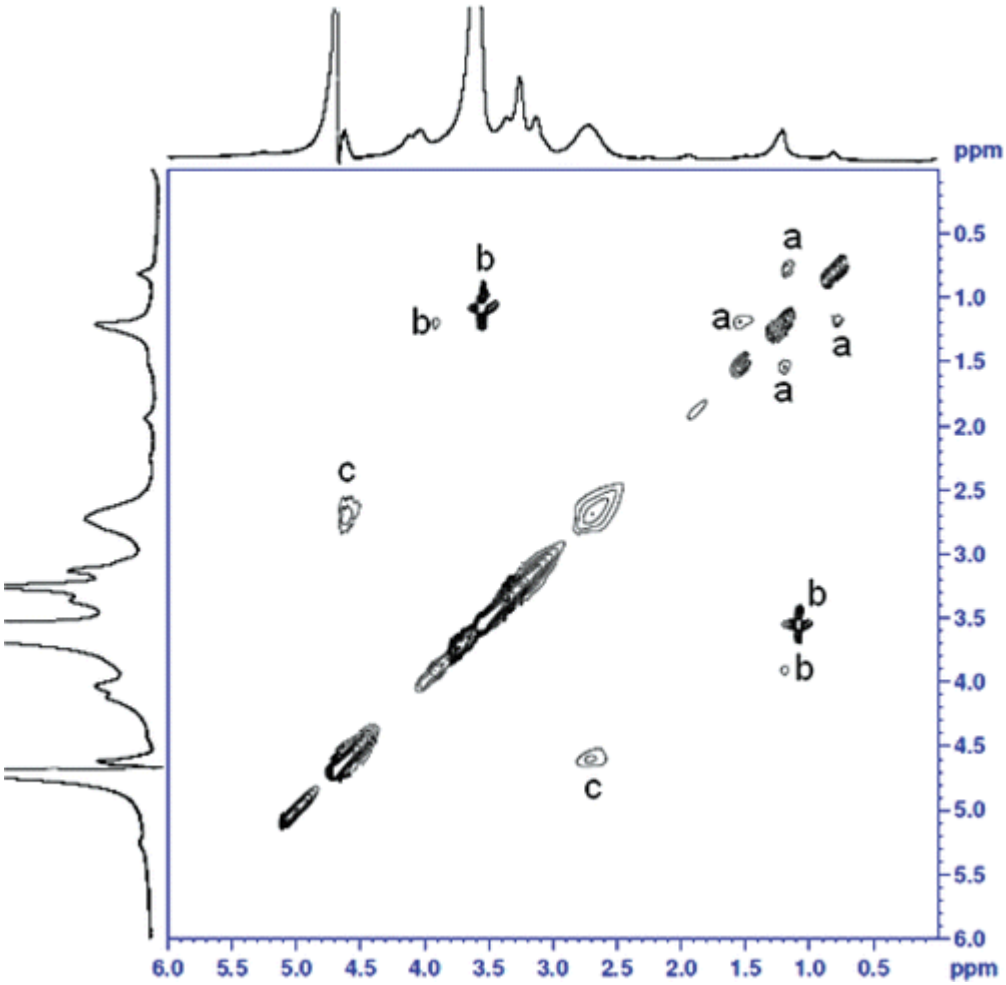


CAC was determined by the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentrations; the estimated value for this system was 0.25 mg mL^{-1}.

Another approach used to study the formation of micellar aggregation systems is the 2D Nuclear Overhauser Effect Spectroscopy (NOESY) technique, which identifies the interactions and relative spatial correlations between protons of copolymer portions forming the micelle structure; this technique has been used to study polymeric micelles formation (Licciardi et al., 2011).

Figure 3 shows the contour plot of the NOESY experiment on PHEA-EDA-Sq_{17}-PS_{80} micelles, performed in D_2O. How it can be seen in the 2 D spectrum, several cross-peaks due to intramolecular and intermolecular correlations can be distinguished. In particular, the cross-peaks (a) between methyl (0.9 ppm) and methylene (1.2–1.5 ppm) protons of Sq_{17} portions were found, these are attributable both to intramolecular and intermolecular correlations between protons of squalenyl chains, forming the micelle core. Particularly important are the cross-peaks between the protons of the polyethylene glycol chains of PS_{80} at 3.6 ppm and methylene protons of Sq_{17} at 1.2–1.5 ppm (b). This is probably due to intermolecular correlation between squalenyl portions, forming the micellar core, and PS_{80} portions in the polymer side chain. This implies that the self-assembling process of PHEA-EDA-Sq_{17}-PS_{80} copolymer molecules, leading to micelles formation, strongly reduces the interfacial width between the hydrophobic core, presumably formed by the hydrophobic squalenyl portions, and the intricately mixed polymeric backbone. Finally, as expected, cross-peaks are also present that can be attributed to the intramolecular correlation between the two different protons of the PHEA backbone (at 2.8 and 4.6 ppm) (c).

Figure 3. Contour plot of the NOESY experiment on PHEA-EDA-Sq_{17}-PS_{80} copolymer micelles recorded with an experimental mixing time of 350 ms.



According to the NOESY theory, the intermolecular cross-peaks observed in the reported spectrum of PHEA-EDA-Sq_{17}-PS_{80} (Figure 3) indicates that the distance between the protons associated with each cross-peak is less than 5 Å. This means that they are close enough to explain the formation of molecular clusters, like micelles, upon interaction of different copolymer molecules (Yang et al., 2009).

Moreover, the formation of small colloidal particles in aqueous media was confirmed by dynamic light scattering measurements. As Table 1 shows, the mean diameter of empty micelles was less than 30 nm, with a narrow size distribution, as shown by the low PDI values.

Table 1. Mean diameter, polydispersity index (PDI) and ζ potential of unloaded and Riv-loaded PHEA-EDA-Sq_{17}-PS_{80} micelles

Sample	Mean diameter (nm)	PDI	ζ Potential (mV ± SD)
Unloaded micelles	28.7	0.26	−18.2±6.35
Riv-loaded micelles	34.1	0.35	−18.1±5.5

Preparation and characterization of rivastigmine-loaded PHEA-EDA-Sq17-PS80 micelles

Rivastigmine is a selective brain carbamate inhibitor of both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), and it is used for the treatment of mild to moderate Alzheimer's disease in adult (Camps and Muñoz-Torrero, 2002; Bachurin, 2003; Williams et al., 2003). Because of the low aqueous solubility of the free base, it is administered as the water-soluble hydrogen tartrate salt, which is rapidly and completely absorbed after oral administration but its bioavailability results almost low (≈36%) with a significant first-pass effect (Williams et a. 2003). Thus, rivastigmine free-base (Riv) was chosen as CNS-active model molecule, in order to evaluate the copolymer's ability to dissolve this molecule with low aqueous solubility and potentially improve its bioavailability.

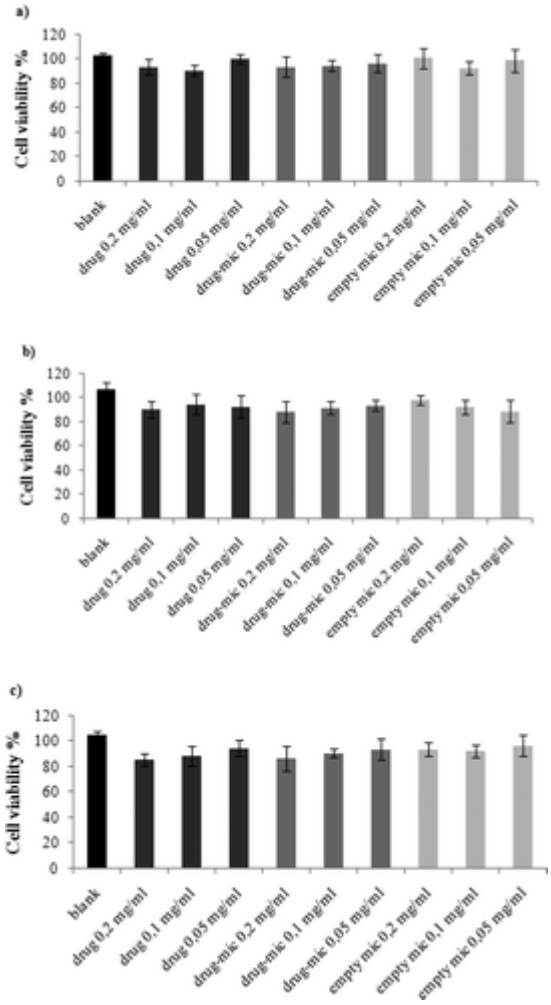
The used drug-loading procedure lead to a good degree of encapsulation; the amount of loaded Riv, evaluated by HPLC (Rao et al., 2005), was 20±0.3% w/w versus the weight of copolymer.

As Table 1 shows, Riv-loaded micelles had a slightly larger diameter than unloaded micelles, probably due to the presence of drug molecules into the micelle core. However, in both cases the particles are small enough to be used for all administration routes, including intravenous. Moreover, the nanoscopic size range of these polymeric micelles might lead to a reduction in their capture by the RES, accumulation in the liver, and rapid elimination from the blood stream (Brannon-Peppas and Blanchette, 2004). Still, unloaded and Riv-loaded micelles have very similar zeta potential values, inferring that dissolved drug molecules are quantitatively enclosed into the micelle core.

In vitro biological evaluations

Cytotoxicity of unloaded and Riv-loaded PHEA-EDA-Sq_{17}-PS_{80} micelles was evaluated by the MTT assay on neuroblastoma (Neuro2a) cells, a cell lines extensively used to screen novel compounds for neurotoxic properties and to investigate the associated mechanisms. These cells were incubated with unloaded and Riv-loaded micelles at concentrations of 1, 0.5 and 0.25 mg mL^{-1}, and with free Riv as positive control at concentrations of 0.2, 0.1 and 0.05 mg mL^{-1} (corresponding to the same concentration of drug loaded into micelles) for 6, 24, and 48 h. The results, in term of cell viability (%) as a function of samples concentration are shown in Figure 4.

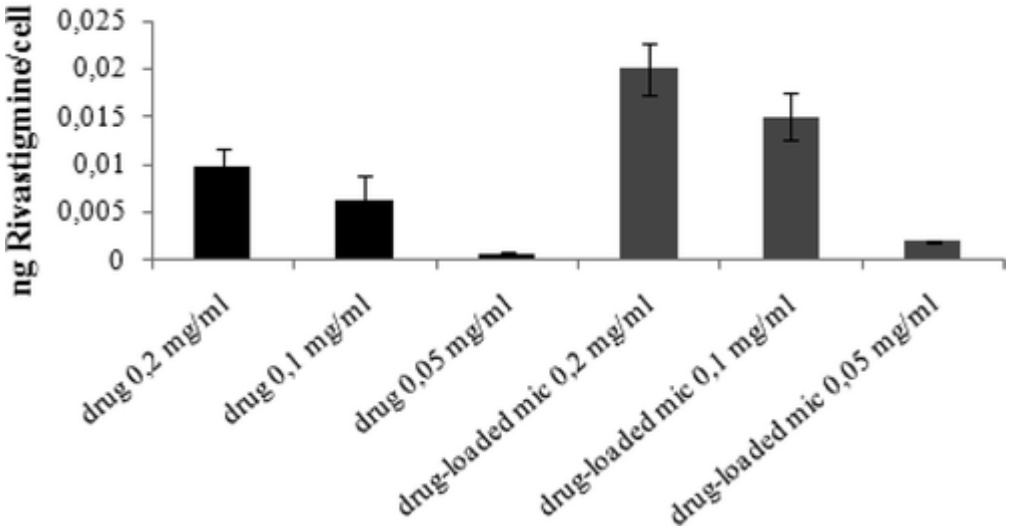
Figure 4. Cell viability of Neuro2a cells incubated for 6 h (a), 24 h (b) and 48 h (c) with free Riv (drug), Riv-loaded micelles (drug-mic) and unloaded micelles (empty mic). Each bar represents the mean of three sample ± SD.



The data show that neither unloaded and nor Riv-loaded micelles have any cytotoxic effect on the tested cells, under the conditions used: cell viability was above 85% for all assay.

In view of the lack of any cytotoxic effects on the neuronal cell-line tested, it was decides to evaluate the potential ability of these micelles to cross neuronal cells membranes, leading to increased drug uptake. For this purpose, Neuro2a cells were incubated with Riv-loaded micelles, at the same drug concentrations used in the cytotoxic experiments, for 6 h. Figure 5 shows the results, in terms of ng of Riv per cell, as a function of drug concentration (free or loaded into micelles). The amount of Riv in cell lysate, detected by HPLC analysis, of samples incubated with Riv-loaded micelles was greater (double) than that found in the cell lysate obtained by incubation with free drug, and as expected drug uptake was dependent on the micelles concentration in the incubation medium. These data suggest that PHEA-EDA-Sq_{17}-PS_{80} micelles may facilitate Riv uptake by neuronal cells, compared with free drug, and that this is probably due to the ability of PS_{80} to transport the carrier across neuronal cell membranes, together with the small mean diameter of the drug-loaded micelles that is less than 33 nm.

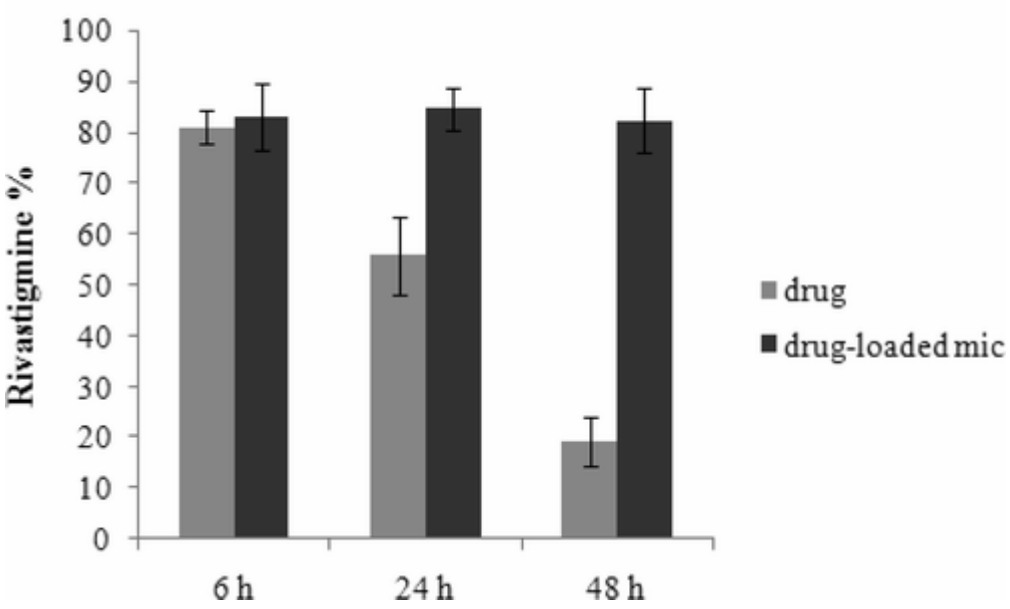
Figure5. Neuro2a uptake of free Riv (drug) and Riv-loaded micelles (drug-loaded mic) after 6h incubation.



Finally, to evaluate the drug's stability in conditions mimicking the physiological environment, an *in vitro* stability study of Riv-loaded micelles was carried out in human plasma. Each sample was incubated with human plasma and drug stability was analyzed by HPLC, evaluating the amount of intact Riv or the presence of drug metabolites.

As Figure 6 shows, the Riv plasma concentration decreased when incubated in the free form; 76% of free Riv was degraded after 48h incubation. Conversely, when Riv was loaded into micelles, about 80% of intact drug was detected after 48h incubation. This means, that the new micelle system is effectively able to halt drug degradation, and that it prevents the loaded drug, presumably located inside the hydrophobic micelle core, from binding with plasma proteins.

Figure 6. Stability profile of Riv-loaded into PHEA-EDA-Sq_{17}-PS_{80} micelles (drug-loaded mic) after incubation in human plasma for 6, 24 and 48 h, in comparison with free rivastigmine (drug).



Conclusions

In this work, a novel amphiphilic graft copolymer has successfully been synthesized starting from the polyaspartamide PHEA, by grafting both polysorbate and C_{17}-squalenyl chains onto the polymeric backbone, obtaining the new copolymer named PHEA-EDA-Sq_{17}-PS_{80}. Copolymer self-assembling was exhaustively studied in aqueous medium by means of 2D ^{1}H-NMR NOESY experiments. This technique showed that the PHEA-EDA-Sq_{17}-PS_{80} copolymer is able to self-assemble into micelle structures in water, and that hydrophobic interaction between Sq_{17} alkyl chains occurs to form the micelle core. A fluorescence probe study showed that the CAC of PHEA-EDA-Sq_{17}-PS_{80} micelles was 0.25 mg mL^{-1}. The micelles had a nanometric hydrodynamic diameter with narrow size distribution and negative surface charge. Moreover, PHEA-EDA-Sq_{17}-PS_{80} micelles were able to incorporate large amounts of the hydrophobic molecule Riv, and the resulting system maintained the stability of the loaded Riv after incubation in human plasma. *In vitro* biological assays evidenced no cytotoxic effects of either unloaded or loaded micelles on the neuronal cell-lines tested, in the applied experimental conditions. Furthermore, the loaded micelles were successfully internalized by the neuroblastoma cell-line, with drug uptake depending on micelles concentration. This appears to indicate that PHEA-EDA-Sq_{17}-PS_{80} micelles are potentially useful as a targeting drug delivery system to the brain.

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