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New self-assembling polyaspartylhydrazide copolymer micelles for anticancer drug delivery

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ABSTRACT

A new amphiphilic copolymer have been synthesized starting from the hydrosoluble polyaspartylhydrazide (PAHy) polymer, by grafting both hydrophilic PEG_{2000} chains and hydrophobic palmitic acid (C_{16}) moieties on polymer backbone, and the structure of obtained PAHy–PEG_{2000}–C_{16} copolymer have been characterized by 2D $^{1}H/^{13}C$ NMR experiments. PAHy–PEG_{2000}–C_{16} copolymer showed the ability of self-assembling in aqueous media giving a core–shell structure and resulted potentially useful for encapsulating and dissolving hydrophobic drug. The formation of micellar core–shell structure has been investigated by 2D ^{1}H NMR NOESY experiments. The presence of cross-peaks for protons of C_{16} and PAHy portions, indicated that the two domains are in close proximity forming micelle core. The critical aggregation concentration (CAC) values of PAHy–PEG_{2000}–C_{16} amphiphilic graft copolymer was determined in water by fluorescence technique, and it was demonstrated that PAHy–PEG_{2000}–C_{16} micelles are well suited to be micellar vehicle of highly hydrophobic molecules. Therefore, anticancer drug tamoxifen, used as a model hydrophobic molecule, was loaded into PAHy–PEG_{2000}–C_{16} micelles obtaining an increase of drug solubility of about 3000 times. Transmission electron microscopy (TEM) observations showed the spherical morphology of micelles formed by PAHy–PEG_{2000}–C_{16} copolymer with a mean diameter of about 30 nm, as confirmed also by dynamic light scattering (DLS) studies. Finally, in vitro cell viability studies were carried out on human breast cancer cells (MCF-7) testing the pharmacological activity of tamoxifen-loaded PAHy–PEG_{2000}–C_{16} micelles, in comparison with free tamoxifen at different drug concentrations, demonstrating that tamoxifen-loaded PAHy–PEG_{2000}–C_{16} micelles exhibited a concentration-dependent cytotoxic activity.

1. Introduction

Today, polymeric micelles occupy a crucial role in the field of drug delivery (Torchilin, 2001, 2002; Matsumura, 2008), thanks to the advantages that they offer in comparison with the administration of drugs in the free form. In principal, micelles are suitable for delivering hydrophobic drugs whose clinical application is limited by their low solubility in aqueous solutions and offer the possibility of prolonging the drug circulation time in the bloodstream (Kwon and Okano, 1996; Torchilin, 2007). Moreover, micelles have dimensions ranging in the range of colloids (nanometers) that allow their administration via parenteral route and permit to delivery high drug amount to solid tumours (Rijcken et al., 2008) by combining synergistically their good drug loading capacity and the passive targeting related to the enhanced permeation and retention (EPR) effect (Iyer et al., 2006).

Respect to low molecular weight surfactants (LMWS), in which micelle formation was originally observed, polymeric micelles show better static and dynamic stability, in consideration of their low CMC values (usually ranging from 10^{-4} to 10^{-7} M), so that polymeric micelles can maintain their self-assembling structure also under highly diluted conditions, for example in the bloodstream, also for long periods of time (Bae and Yin, 2008; Rösler et al., 2001).

Polymeric micelles can be obtained by self-assembling of amphiphilic copolymers in which hydrophilic and hydrophobic portions segregate into two phases in aqueous solutions, assuming a very stable core–shell structure (Voets et al., 2006; Carlsen and Lecommandoux, 2009). The hydrophobic inner core of polymeric micelles can serve as nano-depot for loading molecules with low water solubility, due to hydrophobic interactions between hydrophobic portion of the amphiphilic copolymer and lipophilic molecule (Allen et al., 1999; Savić et al., 2003; Sezgin et al., 2006).

In this paper we report the synthesis and molecular characterization of a new polyaspartylhydrazide (PAHy) based amphiphilic copolymer obtained by chemical conjugation of both poly(ethylene glycol) having a average molecular weight of 2000 g mol^{-1}

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(PEG_{2000}) and palmitic acid (C_{16}) residues on the polymer backbone by a two consecutive reaction steps.

PAHy is a freely water soluble, biocompatible, nontoxic and nonantigenic polymer, which has been used for the synthesis of macromolecular prodrugs (Giammona et al., 1994), for the preparation of polymeric hydrogel for controlled drug delivery (Pitarresi et al., 2000), as polymeric component of lipid supramolecular aggregates (Paolino et al., 2008), and for the preparation of polycations for gene delivey (Cavallaro et al., 2005, 2008). In this paper, the obtained PAHy–PEG–C_{16} copolymers have been used to prepare polymeric micelles with the aim to increase the solubility and cellular uptake of hydrophobic drugs, such as the anticancer tamoxifen, used as model lipophilic drug (Chawla and Amiji, 2002). PEG portions have been chosen for the well known ability of PEG corona to sterically stabilize polymeric micelles, preventing aggregation and formation of large clusters and minimize both non-specific interaction with plasma proteins and interaction with macrophages of reticuloendothelial system (RES) (Kwon and Okano, 1996; Gref et al., 2000). In particular, PEG chains having a molecular weight of 2000 Da are sufficiently longer to form an external hydrophilic shell able to stabilize colloidal systems and confer "stealth" properties to nanodevices for drug delivery (Kwon and Okano, 1996; Gref et al., 2000).

On the other hand, C_{16} portions have been chosen for their ability to give hydrophobic interaction arising the formation of polymeric micelles when linked to other polyaminoacid polymers (Caliceti et al., 2001; Mendichi et al., 2003). The micelle formation have been demonstrated by means of different analysis including fluorescence spectroscopy, light scattering, 2D ^{1}H NMR NOESY experiments and transmission electron microscopy (TEM) observations. All these experiments denoted a core–shell micelle structure of PAHy–PEG–C_{16} aggregates, in which C_{16} hydrophobic portions may be oriented to form the hydrophobic core and reduce distance between PAHy chains, while PEG chains are exposed on the micelle surface and constitute the water-swollen micelle corona, conferring stability and potential stealth properties to micelles.

Finally, in order to evaluate the ability of tamoxifen-loaded micelles to positively affect cell internalization of drug, cell viability studies have been carried out on human breast cancer cells (MCF-7) treating these cells with tamoxifen-loaded micelles in comparison with free drug.

2. Experimental

2.1. Materials

Tamoxifen was purchased from Aldrich Chemical Company. O-[2-(6-Oxocaproylamino)ethyl]-O′-methylpolyethylene glycol 2000 (PEG_{2000}CHO), dichloromethane, dimethyl sulfoxide (DMSO), palmitic acid, N,N′-dicyclohexylcarbodiimide 99% (DCC) and diethyl ether were furnished by Fluka (Switzerland). N-hydroxysuccinimide (NHS), Sephadex G-15, dimethylformamide (DMF), lithium bromide (LiBr) were purchased from Sigma–Aldrich (Italy). PAHy was prepared as previously reported (Giammona et al., 1994).

2.2. Synthesis of PAHy–PEG_{2000}

PAHy (200 mg; 1.55 mmol repeating units, R.U.) was dissolved in 8 ml double distilled water. The pH value of reaction mixture was set at 5 with HCl 0.1N. Then PEG_{2000}CHO (156 mg; 0.078 mmol; molar ratio = 0.05 moles of PEG_{2000}CHO/PAHy R.U.) was added to PAHy solution and reaction was carried out for 20 h at room temperature under continuous stirring. Obtained PAHy–PEG_{2000} copolymer was purified by gel permeation chromatography using Sephadex G-15 as separating gel. After purifications the copolymer solution was freeze-dried from water; the pure product was obtained in 135% yield (w/w) based on starting PAHy. Obtained copolymer was characterized by ^{1}H NMR. ^{1}H NMR (300 MHz, D_2O, 25 °C) δ: 1.54 (m, 4H, –(CO)CH_2–(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 2.25 (m, 2H, –(CO)CH_2–(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 2.68 (m, 2H, –(CO)CH_2–(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 2.78 (m, 2H, CH–CH_2–CO–NH–)$_{PAHy}$, 3.29 (s, 3H, –O–CH_3)$_{PEG}$, 3.42 (m, 4H, –O–CH_2–CH_2–NH–)$_{PEG}$, 3.6–3.7 (s, 174H, –CH_2–CH_2–O–)$_{PEG}$, 3.87 (m, 2H, –O–CH_2–CH_2–NH–)$_{PEG}$, 4.64 (m, 1H, –NH–CH(CO)CH_2)$_{PAHy}$.

2.3. Synthesis of PAHy–PEG_{2000}–C_{16}

Palmitic acid (15 mg; 0,058 mmol) was dissolved in 4 ml of dichloromethane followed by the addition of DCC (13 mg, 0.063 mmol) and NHS (7 mg, 0,061). Reaction mixture was stirred for 20 h at room temperature and than filtered through 0.45 μm Nylon filter. Dichloromethane was evaporated under vacuum and the activated palmitic acid obtained as white powder. A solution of 100 mg of PAHy–PEG_{2000} in 4 ml of DMSO was added dropwise under continuous stirring to activated palmitic acid and reaction was carried out for 20 h at room temperature. Obtained PAHy–PEG_{2000}–C_{16} copolymer was precipitated into diethyl ether and the suspension was centrifuged at 12,000 × g for 10 min at 4 °C. The obtained solid residue was dissolved in double distilled water and purified by exhaustive dialysis using SpectraPor Dialysis Tubing with molecular weight cut-off of 12,000–14,000 Da. After dialysis solution was freeze-dried from water; the pure products was obtained in 90% yield (w/w) based on starting PAHy–PEG_{2000}. Obtained copolymer was characterized by ^{1}H NMR and DEPT-135 ^{13}C NMR analysis.

^{1}H NMR (300 MHz, D_2O, 25 °C) δ: 0.72 (m, –(CH_2)$_{14}$–CH_3) C_{16}; 1.21 (m, 28H, –(CH_2)$_{14}$–CH_3) C_{16}; 1.54 (m, 4H, –(CO)CH_2-(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 2.25 (m, 2H, –(CO)CH_2–(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 2.68 (m, 2H, –(CO)CH_2–(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 2.78 (m, 2H, CH–CH_2–CO–NH–)$_{PAHy}$, 3.29 (s, 3H, –O–CH_3)$_{PEG}$, 3.42 (m, 4H, –O–CH_2–CH_2–NH–)$_{PEG}$, 3.6–3.7 (s, 174H, –CH_2–CH_2–O)$_{PEG}$, 3.87 (m, 2H, –O–CH_2–CH_2–NH–)$_{PEG}$, 4.64 (m, 1H, –NH–CH(CO)CH_2)$_{PAHy}$.

DEPT-135 ^{13}C NMR (300 MHz, D_2O, 25 °C, δ): 13.9 (–(CH_2)$_{14}$–CH_3) 22.68/29.98 (–(CH_2)$_{14}$–CH_3); 20.1 (–(CO)CH_2–(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 25.0/25.3 (–(CO)CH_2–(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 35.1 (–CO–CH–CH_2–CO–NH–)$_{PAHy}$, 35.4 (–(CO)CH_2–(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 36.7 (–(CO)CH_2–(CH_2)$_2$–CH_2–CH=N–)$_{PEG}$, 38.9 (–O–CH_2–CH_2–NH–)$_{PEG}$, 49.5/50.7 (–NH–CH(CO)CH_2)$_{PAHy}$, 58.0 (–O–CH_3)$_{PEG}$, 68.9 (–O–CH_2–CH_2–NH–)$_{PEG}$ 69.6–71.0 (–CH_2–CH_2–O)$_{PEG}$.

2.4. Size exclusion chromatography (SEC) characterization

The weighted average molecular weights and polydispersity values of PAHy and PAHy–PEG_{2000} copolymer were determined by size exclusion chromatography (SEC) in aqueous phase. The standard SEC protocol involved the use of two TSK-GEL columns from TOSOH (G4000PW and G3000PW) connected to a Waters 2410 refractive index detector. Phosphate buffer solution at pH 8,5 was used as eluent at 37 °C with a flux of 0.8 ml/min. SEC analysis for PAHy–PEG_{2000}–C_{16} was performed using two Phenomenex columns; DMF + LiBr 0,1% was used as eluent at 50 °C with a flux of 0.8 ml/min. Poly(ethylene oxide) standards (range 2740–0.5 kDa) were used for calibration in both the protocols.

2.5. NMR spectroscopy characterizations

All ^{1}H and ^{13}C NMR experiments were performed in deuterium oxide (D_2O, Aldrich) and d_7-DMF (only for ^{1}H NMR) solution using

a Bruker Avance II 300 spectrometer operating at 300 MHz. In particular, ^{1}H NMR spectra were recorded using the water suppression method; ^{13}C NMR spectra were recorded as distortionless enhancement by polarization transfer (DEPT) experiments. The DEPT experiment permits to differentiate between CH, CH_2 and CH_3 groups by variation of the selection angle parameter (the tip angle of the final 1H pulse): in this case a 135° angle (DEPT 135) was selected giving a spectra with all CH and CH_3 in positive phase and CH_2 in the opposite phase.

Two-dimensional heteronuclear multiple quantum coherence (HMQC) and nuclear Overhauser effect spectroscopy (NOESY) spectra were recorded as 256 experiments of 2048 data points, using standard Bruker software. The mixing time was varied in the range 250–350 ms.

2.6. Preparation of tamoxifen-loaded micelles

Tamoxifen-loaded micelles were prepared according to a published procedure (Cavallaro et al., 2004). In particular, tamoxifen-loaded micelles were prepared by closely mixing proper amount of PAHy–PEG_{2000}–C_{16} (40 mg) and tamoxifen (10 mg) to obtain a final copolymer/drug weight ratio equal to 4:1. Then, 200 μl of ethanol, and subsequently aliquots of 500 μl of water were added under continuous stirring until reaching 10 ml of volume. The obtained dispersion was sonicated in an ultrasonic bath for 10 min and left overnight under continuous stirring. Finally, dispersion was homogenized with Ultra-Turrax, centrifuged at 11,800 rpm, at 25 °C for 10 min, filtered on cellulose acetate 0.22 μm and freeze-dried.

2.7. Determination of drug content

Tamoxifen loading capacity of micelles was determined by a HPLC method using a reversed-phase C_{18} column as stationary phase and CH_3OH/0.1 M K_2HPO_4 (pH 8.7) (90/10, v/v) at the flow rate of 1.5 ml/min as mobile phase. The eluate was monitored at the wavelength of 250 nm. 5 mg of freeze-dried micelles were dissolved in 2 ml of distilled water. The aqueous solutions, filtered through 0.45 μm cellulose acetate membrane filters, were analyzed by HPLC. Results were expressed as weight percent of drug amount in 100 mg of dried material.

2.8. Determination of critical aggregation concentration (CAC) of PAHy–PEG_{2000}–C_{16} copolymer

The CAC for PAHy–PEG_{2000}–C_{16} was determined by fluorescence spectroscopic analysis, using pyrene as a hydrophobic fluorescent probe. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorophotometer. Aliquots of pyrene solutions (6×10^{-5} M in acetone, 20 μl) were added into vials. After evaporation of acetone in orbital shaker at 35 °C, 2 ml of aqueous copolymer solutions at concentrations ranging from 1×10^{-4} to 10 mg/ml were then added to the vials containing the pyrene residue; the final concentration of pyrene was 6.0×10^{-7} M in each sample. The solutions were kept at 35 °C for 30 min to reach the dissolution equilibrium of pyrene in the aqueous phase, and then left to cool overnight at room temperature to ensure equilibration between the pyrene within the micelles and that in the aqueous solution. Pyrene excitation and emission spectra were recorded at 25 °C using an emission wavelength of 373 nm and an excitation wavelength of 333 nm.

2.9. Dynamic light scattering (DLS) studies and Z-potential measurements of PAHy–PEG_{2000}–C_{16} micelles

DLS studies and Z-potential measurements were performed at 25 °C using a Malvern Zetasizer NanoZS instrument, fitted with a 532 nm laser at a fixed scattering angle of 90°. The PAHy–PEG_{2000}–C_{16} copolymer was molecularly dissolved in double distilled water (pH 6) at concentrations ranging from 1×10^{-4} to 10 mg/ml and the intensity-average hydrodynamic diameter (size in nm) and polydispersity index (PDI) were obtained by cumulant analysis of the correlation function. The Z-potential (mV) was calculated from the electrophoretic mobility using the Smoluchowsky relationship and assuming that $Ka \gg 1$ (where K and a are the Debye–Hückel parameter and particle radius, respectively). Each experiment was performed in triplicate.

2.10. Transmission electron microscopy analyses

Samples for TEM observations were prepared settling a drop of 0.1% (w/v) sample solutions containing micelles, in distilled water, on the copper grid. The excess of sample was wicked away with the aid of filter paper and the solution on the grid was allowed to dry spontaneously.

The samples were analyzed using TEM (JEM-2100 LaB_6 transmission electron microscope operating at an accelerating voltage of 200 kV, equipped with a MultiScan CCD camera) in order to evaluate micelle size and shape.

2.11. Cytotoxicity assay

MCF-7 breast cancer cells were purchased at Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Italy. Cells were seeded in a 96 well plate at a density of 5×10^4 cells/well and grown in Minimum essential medium (MEM) with 10% FBS (foetal bovine serum) and 1% of penicillin/streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin), at 37 °C in 5% CO_2 humidified atmosphere.

After 72 h, cells were treated with tamoxifen-loaded micelle solutions in bidistilled water, having a tamoxifen concentration ranging from 10^{-7} to 10^{-4} M. Aliquots of micelle solutions (10 μl) were added to the cells in 100 μl fresh medium and incubated for 48 h. After incubation, 20 μl of MTT reagent solution (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; 0.5 mg/ml) were added to each well and plates were incubated at 37 °C for 2 h; than the absorbance was measured by multiwell plate reader (Multiskan Ex, Thermo Labsystems, Finlandia), at 490 nm after background correction. Cells treated with tamoxifen solutions in DMSO, with a tamoxifen concentration ranging from 10^{-7} to 10^{-4} M, were used as positive control; untreated cells as negative control.

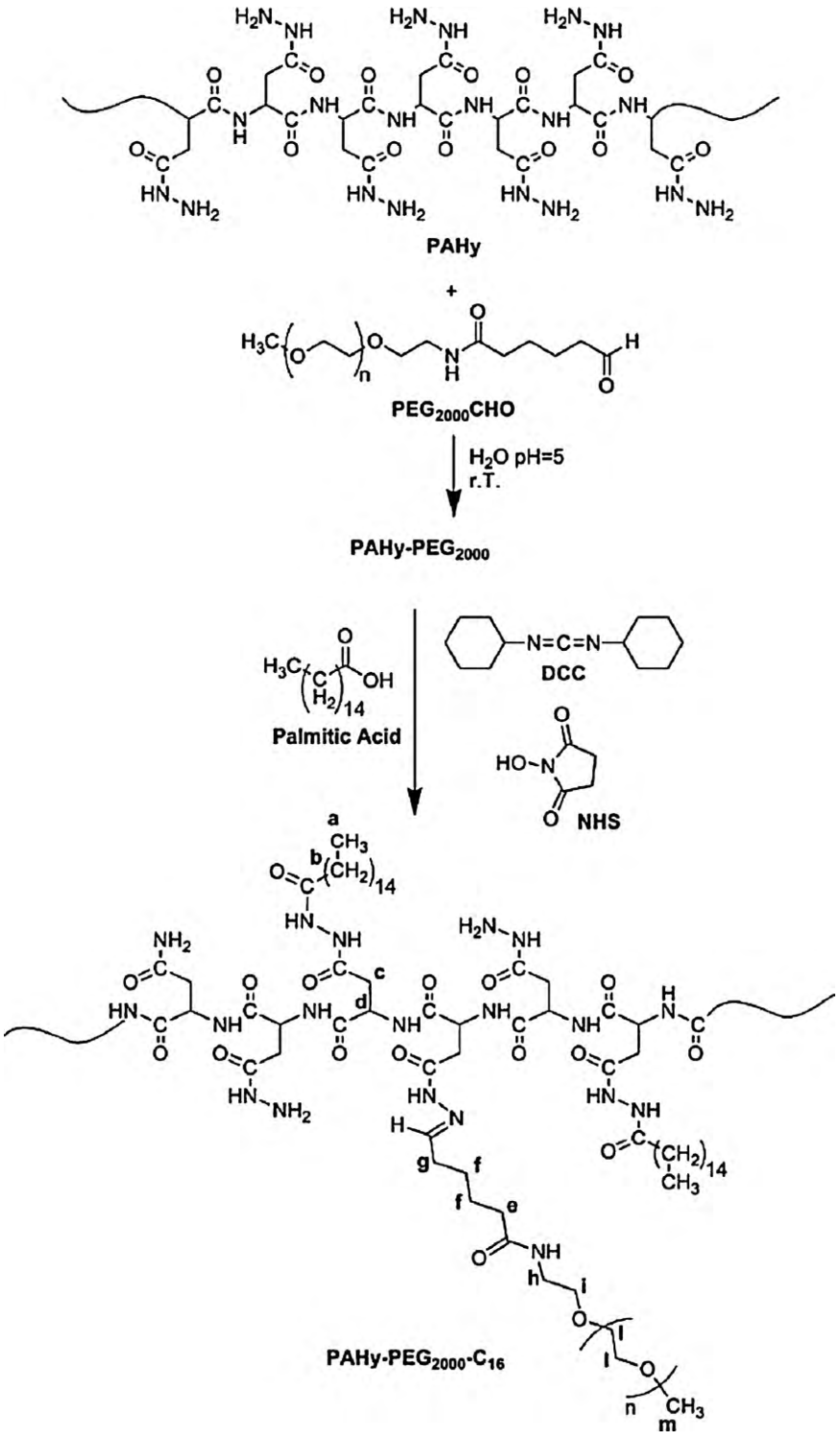
2.12. Statistical analysis

A one-way analysis of variance (ANOVA) was used to evaluate group comparison. If the group by each time interaction was significantly different ($P < 0.05$), differences between groups were compared within a posteriori Bonferroni t-test. All the values are reported as the average ± standard deviation.

3. Results and discussion

3.1. Synthesis of PAHy–PEG_{2000} and PAHy–PEG_{2000}–C_{16} copolymers

The synthesis of PAHy–PEG_{2000}–C_{16} copolymer was performed starting from PAHy by two subsequent reactions involving firstly



Scheme 1. Synthesis of PAHy–PEG_{2000}–C_{16} copolymer (n_{PEG} = 44).

the conjugation of PEG chains and secondly the attachment of C_{16} portions. PEG chains were grafted on the PAHy backbone by a simple reaction with an aldehyde functionalized mono-methoxy PEG with an average molecular weight of 2000 Da.

Aldehyde functional group easily reacted with hydrazidic $-NH_2$ of PAHy in slightly acidic aqueous medium; indeed, reaction between PAHy and aldehyde functionalized mono-methoxy PEG yielded to a quantitative conjugation of added PEG chains to PAHy (see Scheme 1).

PAHy–PEG_{2000} copolymer was purified and characterized through ^{1}H NMR spectroscopy, which confirmed the introduction of PEG_{2000} chains on the PAHy backbone and permitted the calculation of molar derivatization degree (DD_{PEG}%). The DD%, indicated as percentage of linked polyethylene chains in comparison with repeating units of PAHy, was calculated by comparing the integral of the peak related to protons at δ 3.67 assigned to $(-CH_2-CH_2-O-)_n$ belonging to PEG with the integral of the peaks related to protons at δ 2.78 assigned to –NH–CH(CO)CH_2– belonging to PAHy back-

bone. The molar percent of PEG chain covalently linked to PAHy was equal to 5% (mol/mol) respect to polymer repeating units.

Subsequently, the activation reaction of carboxylic group of palmitic acid with DCC and NHS followed by the reaction with PAHy–PEG_{2000} copolymer allowed the conjugation of hexadecyl pendant chains, obtaining the PAHy–PEG_{2000}–C_{16} copolymer, whose structure is shown in Scheme 1.

The DD_{C16}%, indicated as percentage of hexadecyl chains respect to repeating units of PAHy, was calculated by comparing the integral of the peak related to protons at δ 0.72 assigned to $-CH_2-CH_3$ (or the integral of the peak related to protons at δ 1.28 assigned to $-(CH_2)_{14}-CH_3$) that belong to linked C_{16} moiety, with the integral of the peak related to protons at δ 2.78 assigned to –NH–CH(CO)–CH_2– belonging to PAHy. The DD_{PEG}% and DD_{C16}% were also calculated by ^{1}H NMR spectra recorded in d_7-DMF because in this solvent aggregation phenomena of the copolymer can be negligible even at copolymer concentration above the CAC, as confirmed by SEC analysis, and thus influence on the intensity of the hydrophobic segment peaks can be excluded.

Using the experimental conditions here reported, a copolymer with a DD_{C16}% equal to 9% was obtained. Preliminary studies showed that a copolymer with a lower DD_{C16}% was obtained by using lower palmitic acid amount for the synthesis of PAHy–PEG_{2000}–C_{16}. For example, by using 0.029 mmol of palmitic acid (1/2 of that reported in Section 2) a copolymer with a DD_{C16}% of 4.5% was obtained. This copolymer, however, was not able to efficiently self-assemble in polymeric micelles. On the other hand by using 0.087 mmol of palmitic acid a copolymer at DD_{C16}% equal to 12% was obtained, but this copolymer was practically water insoluble. Therefore, the copolymer PAHy–PEG_{2000}–C_{16} at DD_{C16}% equal to 9% (mol/mol), showing good water solubility and self-assembling properties, was chosen to be tested as drug delivery system.

3.2. Molecular characterization of the copolymers

The chemical structure of PAHy–PEG_{2000}–C_{16} copolymer was exhaustively characterized by two-dimensional HMQC NMR spectroscopy (Jacobsen, 2007). HMQC is a two-dimensional inverse H, C correlation technique that allows for the determination of carbon (or other heteroatom) to hydrogen connectivity. HMQC, in particular is selective for direct C–H coupling. With this technique, cross-peaks appear as contour relief plots at the intersection of directly coupled C–H peaks. Only directly bonded hydrogen and carbons will give cross-peaks (quaternary carbons are not seen in HMQC), which makes interpretation rather straight foreword. As shown in the two-dimensional spectrum of PAHy–PEG_{2000}–C_{16} (see Fig. 1), assignment was made by drawing two lines at a right angle from each peak of the ^{1}H spectrum (plotted on the horizontal axis) to the correspondent peak of ^{13}C spectrum (plotted on the vertical axis) through the cross-peak, which looks like a series of concentric spots.

The assignments of all peaks was made using the standard Bruker software on the basis of these spectra, and ^{1}H and ^{13}C chemical shifts are reported in Table 1.

Weighted average molecular weights (M_w) and polydispersity index of synthesized copolymers were determined by SEC analyses in aqueous solvent for PAHy and PAHy–PEG_{2000} copolymer, where these polymers result freely soluble, while for PAHy–PEG_{2000}–C_{16}, in order to avoid aggregation phenomena, organic solvent (DMF) was used (see Table 2).

As expected, in fact, SEC chromatogram of PAHy–PEG_{2000}–C_{16} copolymer in aqueous phase showed a peak corresponding to 173 kDa attributed to the formation of micelle aggregates, while there was no evidence of peaks related to copolymer monomers, suitable for M_w determination. This result is consistent with the fact that sample concentration used for this analysis was always above the CAC. The average molecular weights (M_w) of PAHy–PEG_{2000} and PAHy–PEG_{2000}–C_{16} were, respectively, 42.1 and 44.7 kDa. Definitively, the molecular weight increment of PAHy–PEG_{2000} (determined using aqueous solvent) and PAHy–PEG_{2000}–C_{16} (determined using organic solvent, i.e. DMF), are in accordance with the derivatization of PAHy with PEG_{2000} chains and subsequently of PAHy–PEG_{2000} with palmitic acid.

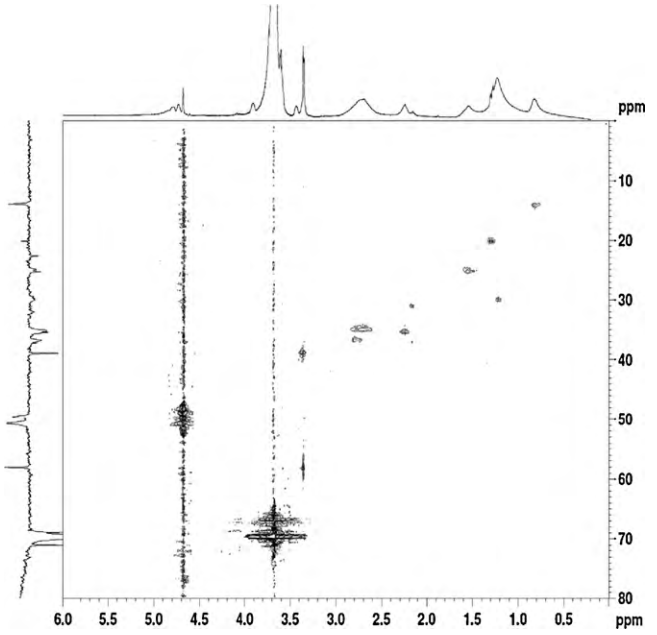


Fig. 1. Two-dimensional HMQC NMR spectroscopy of PAHy–PEG_{2000}–C_{16} copolymer. Top: ^{1}H NMR (D_2O) spectrum of PAHy–PEG_{2000}–C_{16} recorded using the water suppression method; left: DEPT 135 ^{13}C NMR of PAHy–PEG_{2000}–C_{16}.

3.3. Study of the spatial extent of core and corona chains of micelles

The introduction of a proper molar percentage of hexadecyl chains into the water soluble PAHy–PEG_{2000} copolymer, provided an amphiphilic copolymer capable of self-assemble into supramolecular structures in aqueous media, namely direct polymeric micelles, due to hydrophobic interactions between alkyl chains (Yokoyama et al., 1990; Gref et al., 1994). In this structure, the spatial disposition of hydrophobic chains very closely, generated an hydrophobic core in aqueous medium that is potentially

Table 1
Chemical shift assignment (protons and carbons) for PAHy–PEG_{2000}–C_{16} copolymer structure.

Proton/carbon[a]	Chemical shifts (δ) in ppm	
	^{1}H	^{13}C[b]
a	0.72	13.9
b	1.21	22.6/29.9
c	2.78	35.1
d	4.64	50.7
e	2.68	36.7
f	1.54	25.3
g	2.25	35.4
h	3.42	38.9
i	3.87	68.9
l	3.6–3.7	69.6
m	3.35	58.0

[a] Proton and carbon assignments are indicated in PAHy–PEG_{2000}–C_{16} structure of Scheme 1.
[b] Quaternary carbons are not reported because spectrum was recorded as DEPT 135 ^{13}C NMR.

Table 2
Values of the main physico-chemical characteristics of PAHy copolymers obtained by SEC and ^{1}H NMR analysis.

Polymer	M_w (kDa)	Polydispersity (M_w/M_n)	DD[a] $_{PEG2000}$ (mol%)	DD[a] $_{C16}$ (mol%)
PAHy	28.7[b]	1.7	–	–
PAHy–PEG_{2000}	42.1[b]	1.8	5%	–
PAHy–PEG_{2000}–C_{16}	44.7[c]	1.7	5%	9%

[a] DD: derivatization degree, calculated by means of ^{1}H NMR.
[b] The M_w value was determined using aqueous solvent for SEC analysis.
[c] The M_w value was determined using organic solvent (DMF) for SEC analysis.

able to entrap lipophilic molecules. Several approaches have been used to study the formation of micelle core, such as fluorescent probe techniques (Lavasanifar et al., 2001). In this paper two-dimensional ^{1}H NMR NOESY experiments were performed to study the formation of micelle core by identifying the interactions and relative spatial correlations between protons of copolymer domains involved in the micelle core formation (Voets et al., 2006). NOESY is a well known two-dimensional NMR technique probing internuclear distances by means of the nuclear Overhauser effect (NOE). This effect describes the change in resonance intensity of a proton due to saturation of another proton in the vicinity, and depends on the fraction of spin-lattice relaxation (T1) of the first proton caused by its dipolar interaction with the second proton (Voets et al., 2006; Mo and Pochapsky, 1997; Atkins, 2002; Wüthrich, 1986). The results of a 2D ^{1}H NMR NOESY experiment are typically presented in a so-called contour plot, where the one-dimensional ^{1}H NMR spectra are plotted on the vertical and horizontal axis. When a proton A interact with a proton B, a so-called cross-peak appears on the intersection of two straight lines at the chemical shifts of protons A and B. If protons A and B differ in chemical shift, their cross-peak is necessarily off-diagonal, but symmetrical with respect to the diagonal of the 2D spectrum. How it can be seen in the NOESY contour plot of PAHy–PEG_{2000}–C_{16} (see Fig. 2), protons that are in close proximity (typically less than 0.5 nm) generated a symmetric off-diagonal cross-peak.

Observing the 2D spectrum, several cross-peaks can be clearly distinguished due to intramolecular and intermolecular correlations. In particular, either intramolecular or intermolecular correlations were found between protons of hexadecyl chains, accordingly with the pronounced cross-peaks (rhombus) between methyl (0.7 ppm) and methylene (1.2 ppm) protons of C_{16} portions. This result is consistent with the close spatial correlation due to hydrophobic interactions between C_{16} domains in the micelle core. Particularly visible are the cross-peaks attributed to the intramolecular correlation between the two different protons of PAHy backbone (at 2.8 and 4.6 ppm) (ellipses). Interestingly, intermolecular cross-peaks appeared also between C_{16} domain and the above mentioned protons of PAHy backbone (circles). This implies that PAHy chains are intricately mixed, upon micelle formation, and that the interfacial width between hydrophobic core and polymer backbone is very narrow. This interpretation should be better schematized in Fig. 3.

Finally, even if less pronounced, but still visible, there are the cross-peaks between the protons of PEG chains at 3.6 ppm and methylene protons of C_{16} portions at 1.2 ppm (squares), probably due to intermolecular correlation between hexadecyl residues bended to the core surface and in close proximity of polyethylene glycol chains forming the micelle corona. This interpretation is in agreement with that already reported in other NOESY experiments by Gjerde et al. (1996) in the case of sodium dodecyl sulfate/PEO mixed micelles.

3.4. Hydrophobic association study and CAC determination probed by pyrene

Micelles formation was further investigated by means of fluorescence studies using pyrene as fluorescent probe. The fluorescent probe technique is a very sensitive method for detecting the formation of polymeric micelles (Ma et al., 2003; Wilhelm et al., 1991). Due to his hydrophobic nature, pyrene preferentially segregate in the inner core of micelles; as a consequence of transfer from polar to nonpolar environment, the I_1/I_3 of pyrene changes from ~1.8 in water to ~0.6 in nonpolar solvents such as hexane (Chen et al.,

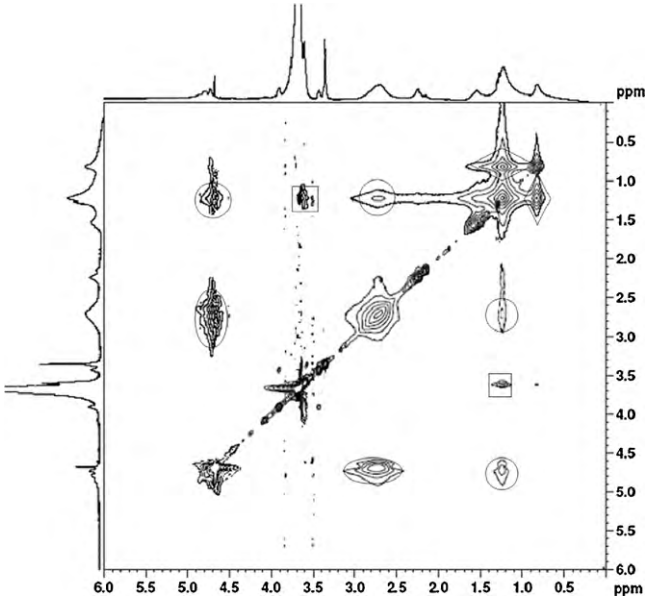


Fig. 2. Contour plot of the NOESY experiment on PAHy–PEG_{2000}–C_{16} copolymer micelles recorded with an experimental mixing time of 350 ms.

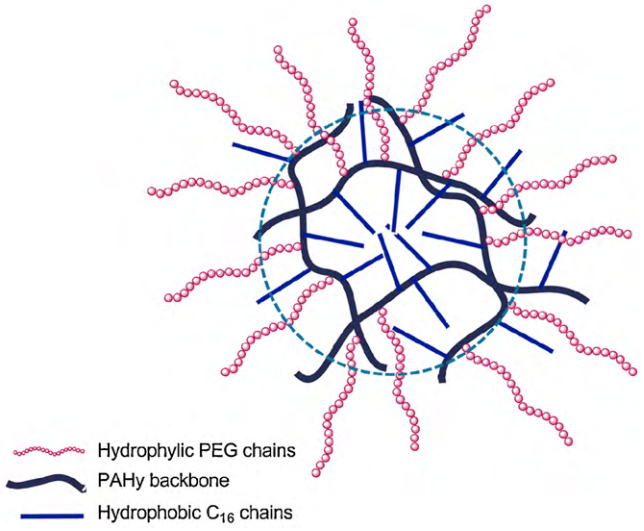


Fig. 3. Schematic representation of polymer chain mixing in the core and corona of PAHy–PEG_{2000}–C_{16} micelles.

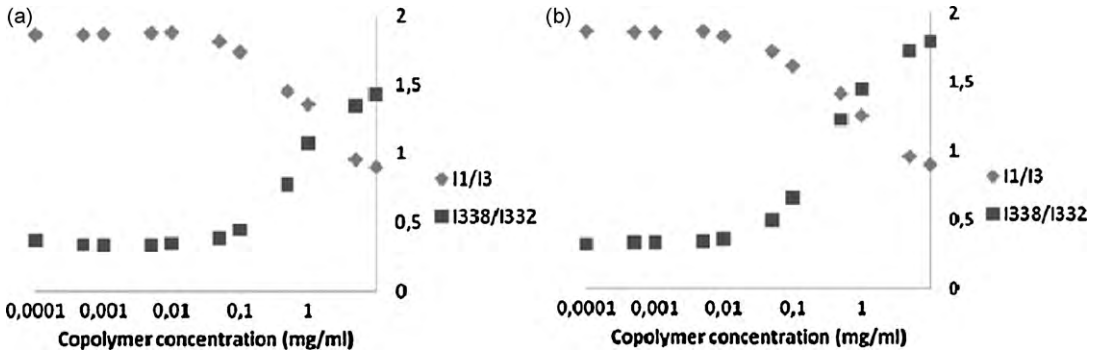


Fig. 4. I_{338}/I_{332} intensity ratios calculated from pyrene excitation spectra (closed squares) and I_1/I_3 intensity ratios obtained from pyrene emission spectra (closed rhombuses) as a function of concentration for (a) empty micelles and (b) tamoxifen-loaded micelles.

1999). Plotting both the I_{338}/I_{332} ratio, obtained from the excitation spectra, and the I_1/I_3 ratio obtained from the pyrene emission spectra recorded at 25 °C, versus the logarithm of the copolymer concentration, the two curves (Fig. 4) suitable for CAC determination were drawn. The CAC was taken from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at lower concentrations. The estimated CAC value was 1×10^{-2} mg/ml for empty micelle and approximately 9×10^{-2} mg/ml for tamoxifen-loaded micelle. The higher CAC value for drug-loaded system is probably due to the interference of tamoxifen molecules on the pyrene emission and excitation properties. The presence of the hydrophobic tamoxifen molecules in the micelle core may induce the exclusion of a part of pyrene molecules from the micelle core or prolong the equilibration time, with the consequent reduction of pyrene emission intensity (Chen et al., 1999; Cao et al., 1991). This fact is also consistent with the preferential dislocation of drug molecules into the micelle core.

3.5. Dynamic light scattering studies

The CAC value for empty micelles was further confirmed by DLS studies at 25 °C, by analyzing samples prepared at the same concentration used for fluorescence studies. In Fig. 5, hydrodynamic diameter (d) and scattering intensity (I) were plotted versus copolymer concentration.

As it can be seen both curves show similar trend; in fact, d and I values gradually increase until reaching a peak value at 1×10^{-2} mg/ml, which exactly corresponds to the CAC value of micelles, as determined by fluorescence studies. At copolymer concentrations above CAC the hydrodynamic diameter of micelles decreased rapidly and stabilize at about 30 nm. The peak value for the hydrodynamic diameter observed at the CAC for empty micelles is in accordance with Gao and Eisenberg (1993); also the decrease of micelle size above this point is in accordance with that work.

In fact, in proximity of CAC, amphiphilic copolymers begin to self-assemble in micellar structures entrapping large quantities of solvent inside the inner core, so resulting as aggregates with larger sizes in comparison with micelles formed at copolymer concentrations above the CAC. Since polymer concentration increases, solvent molecules will be spread out and micelle cores rearrange assuming their lower energy state with a subsequent size equilibration (Gao and Eisenberg, 1993; Halperin and Alexander, 1989). The same results in terms of d and I were obtained repeating DLS analysis of empty micelles after 24 h at 25 °C in double distilled water.

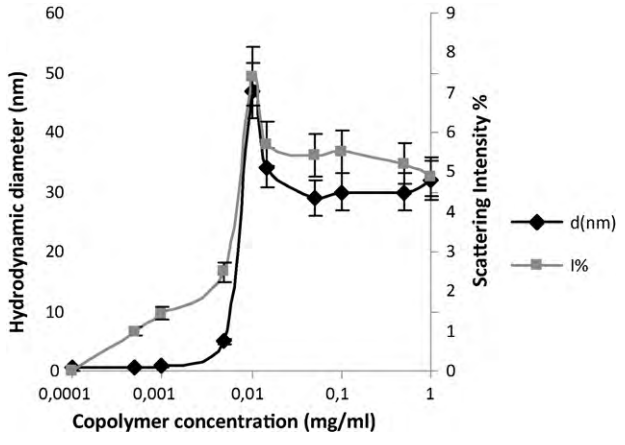


Fig. 5. Hydrodynamic diameter (closed rhombuses) and scattering intensity % (closed squares) of PAHy–PEG_{2000}–C_{16} micelles as a function of copolymer concentration. Data represent mean ± SD ($n = 3$).

3.6. Study of the morphology of micelles

The investigation on micelle morphology was carried out by TEM analysis on samples at 1 mg/ml copolymer concentration, and evidenced that PAHy–PEG_{2000}–C_{16} copolymer forms spherical micelles in water with a diameter of about 30 nm (see Fig. 6). Moreover, TEM images showed two discrete areas with distinct colour intensities due to different optical density, highlighting the formation of a core–shell type structure (Lee et al., 2008).

3.7. Tamoxifen-loaded micelles

In order to evaluate the copolymer ability of dissolving hydrophobic molecules in water, dissolution studies were conducted using tamoxifen as model lipophilic molecule. Tamoxifen, an anticancer hydrophobic drug with a very low solubility in aqueous media (0.04 μg/ml) (Gao and Singh, 1998), was dissolved in water by virtue of the PAHy–PEG_{2000}–C_{16} self-assembling ability into micelles. Drug loading into polymeric micelles was carried out according with a procedure reported in an our previous study (Cavallaro et al., 2004), involving a preliminary mixing of scheduled amounts of drug and copolymer at the dry state; subsequent addition of ethanol caused the formation of a pulpy mixture, in which tamoxifen is partially dissolved. Finally, progressive additions of water aliquots allow self-assembling of copolymer in micellar aggregates and the subsequent incorporation of drug molecules into the hydrophobic micelle core. After equilibration, drug excess was removed from the system by centrifugation and filtration on 0.22 μm filters, that also allowed the sterilization of the micelle solution. The amount of tamoxifen loaded was then determined by HPLC; PAHy–PEG_{2000}–C_{16} micelles were able to incorporate tamoxifen amounts equal to 4% w/w respect to the whole copolymer

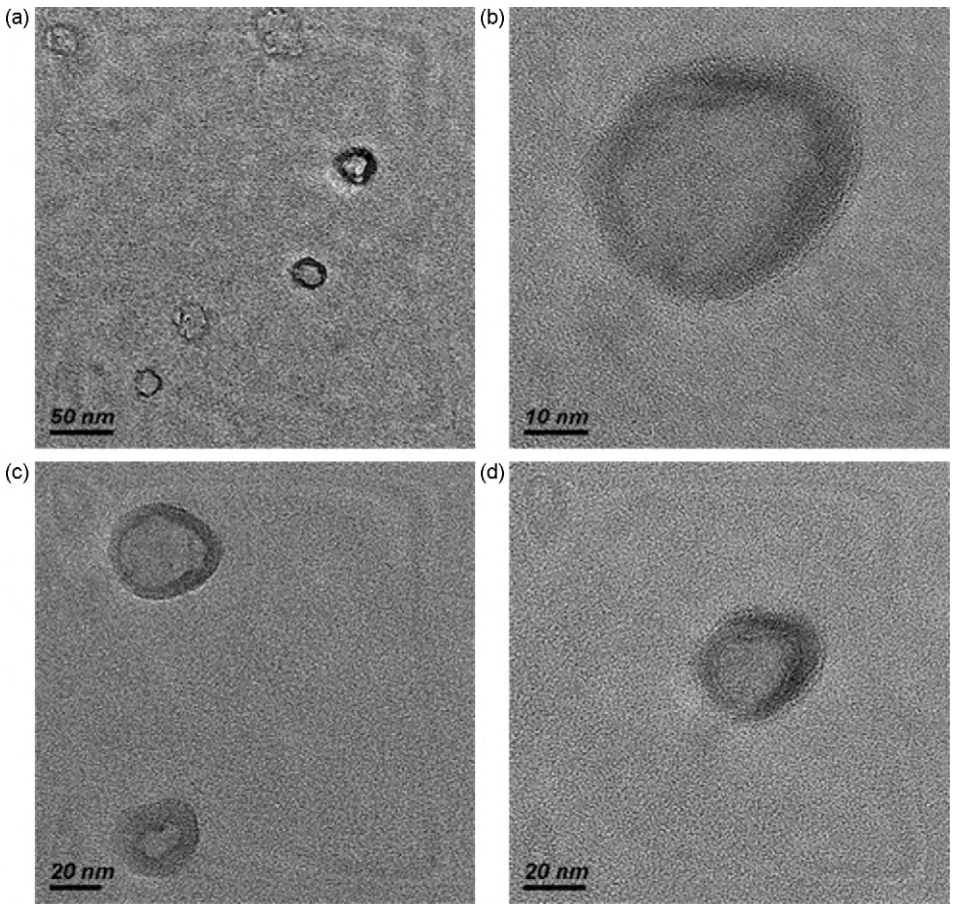


Fig. 6. TEM images of PAHy–PEG_{2000}–C_{16} micelles at the copolymer concentration of 1 mg/ml.

weight and permitted to dissolve in water 0.12 mg/ml of tamoxifen. This value corresponds to an increase of the drug water solubility of about 3000 times. In comparison with the results previously published with an other polyaspartic copolymer bearing the same kind of hydrophilic and hydrophobic portions, such as PHEA–PEG_{2000}–C_{16} (Cavallaro et al., 2004), PAHy–PEG_{2000}–C_{16} micelles resulted threefold more efficient in terms of drug loading. Moreover, the variation of zeta potential values between empty micelles (−2.73 mV) and tamoxifen-loaded micelles (−10.1 mV) suggests that drug molecules may be dissolved not only within the micelle cores but also partially close to the micelle periphery, as elsewhere reported (Licciardi et al., 2006). However, we cannot exclude the possibility that a change in the aggregation number of the copolymer micelles due to drug dissolution may influence the whole micelle zeta potential as well. Finally, dimension, size distribution and morphology of tamoxifen-loaded micelles resulted practically unvaried respect empty micelles and the same results in terms of *d* and *I* were obtained repeating DLS analysis after 24 h.

3.8. Cytotoxicity assay

In order to evaluate the cytotoxic activity of both unloaded and tamoxifen-loaded PAHy–PEG_{2000}–C_{16} micelles, MCF-7 cells were incubated with solutions containing empty micelles (named pol in Fig. 7) or tamoxifen-loaded micelles (named mic in Fig. 7), having the same copolymer concentration. In this study, free tamoxifen samples (named tam in Fig. 7) and DMSO (used to dissolve tamoxifen) were used as positive control. After incubation with the above mentioned samples for 48 h, cells were analyzed by MTT colorimetric assay to evaluate cell viability. As it can be seen in Fig. 7, empty micelles never showed a cell viability below 80% even at the highest micelle concentration (pol 10^{-4}; 0.925 mg/ml) showing an high cytocompatibility of these copolymers in the micelle form. On the contrary, at 10^{-4} M both free (tam 10^{-4}) and encapsulated tamoxifen (mic 10^{-4}) exhibited high cytotoxic effect (0% and 6% viable cells, respectively). Below this drug concentration (tam 10^{-5}, tam 10^{-6} and tam 10^{-7} M) free tamoxifen show a plateau of its cytotoxic activity (64% viable cells), that seems to be partially imputable also to the presence of DMSO (Pommier et al., 1988), as indicated by the toxic affect provided by this solvent (DMSO 2 μl) on MCF-7 cell line in this experiment. Actually, this result proved a high sensitivity of MCF-7 cell versus DMSO when treated with the same DMSO amounts used to dissolve free tamoxifen (72% viable cells). Interestingly, tamoxifen-loaded PAHy–PEG_{2000}–C_{16}

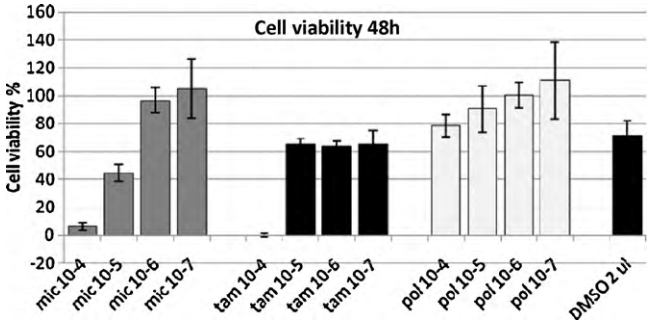


Fig. 7. Cell viability of MCF-7 breast cancer cells incubated for 48 h with free tamoxifen (tam), tamoxifen-loaded micelles (mic), empty micelles (pol) and DMSO (DMSO). Each bar represents the mean of three samples ± SD.

micelles exhibited a concentration-dependent cytotoxic activity; in particular, the micelle sample corresponding to 10^{-5} M tamoxifen concentration (mic 10^{-5}) showed a cell growth inhibition (45% viable cells) more pronounced respect to free tamoxifen at the same concentration (tam 10^{-5}); this effect may probably be imputable to an increased drug permeation into cells mediated by the amphiphilic PAHy–PEG_{2000}–C_{16} graft copolymer. The ability of amphiphilic copolymers at polyaminoacid structure and bearing the same hydrophilic and hydrophobic groups to improve drug cell permeation has been already evidenced by authors (Civiale et al., 2009).

4. Conclusions

In this work, a new amphiphilic graft copolymer have been successfully synthesized starting from the polyaspartylhydrazide PAHy, by grafting both palmitic acid and mono-methoxy polyethylene glycol 2000 on polymer backbone, obtaining the copolymer named PAHy–PEG_{2000}–C_{16}. PAHy–PEG_{2000}–C_{16} self-assembling properties were exhaustively studied in aqueous medium by means of two-dimensional ^{1}H NMR NOESY experiments and fluorescence analysis using pyrene as fluorescent probe. These techniques demonstrated that PAHy–PEG_{2000}–C_{16} copolymer is capable of self-assemble into micellar structures in water, due to hydrophobic interactions between alkyl chains. In particular, NOESY study permitted to identify the interactions and relative spatial correlations between protons of copolymer domains involved in the micelle core formation and thus to understand that micelle core is principally generated by the very close spatial disposition of the hydrophobic palmitic chains and PAHy backbone of PAHy–PEG_{2000}–C_{16} copolymer. Fluorescence probe study permitted to determine the CAC of PAHy–PEG_{2000}–C_{16} micelles that resulted equal to 1×10^{-2} mg/ml. Prepared micelles showed spherical form with a hydrodynamic diameter of about 30 nm as confirmed by TEM and DLS analysis. Moreover, PAHy–PEG_{2000}–C_{16} micelles resulted able to dissolve a great amount of the hydrophobic drug tamoxifen, increasing the hydrosolubility of this drug of about 3000 times. In vitro cytotoxicity studies evidenced that tamoxifen-loaded micelles showed a cell growth inhibition dependent by drug concentration and in one case more pronounced respect to free tamoxifen at the same concentration (tam 10^{-5}). This result let us suppose that PAHy–PEG_{2000}–C_{16} micelles may increase drug permeation into tested cells and make these micelle suitable as colloidal drug delivery systems for lipophilic anticancer drugs.

Acknowledgments

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