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Interaction between Drug Loaded Polyaspartamide-Polylactide-Polysorbate Based Micelles and Cell Membrane Models: A Calorimetric Study

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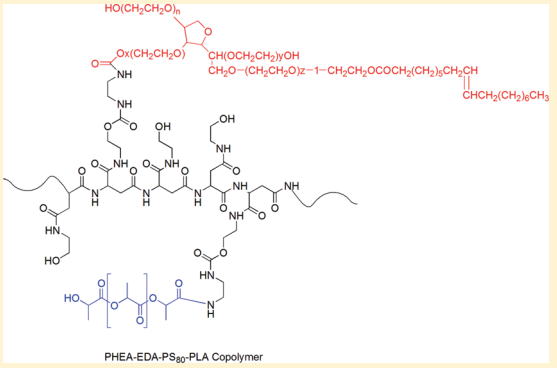
ABSTRACT: Amphiphilic biodegradable copolymers, for their ability to self-assemble into micelle-like aggregates, with a suitable loading capacity, are of emerging interest for the delivery of water-insoluble drugs. α,β-Poly[(*N*-hydroxyethyl)-DL-aspartamide] (PHEA) is suitable to obtain amphiphilic graft copolymers. These copolymers can be obtained starting from PHEA-ethylenediamine (PHEA-EDA) which is functionalized with polysorbate 80 (PS_{80}, like targeting residues to the brain) and polylactide (PLA, like hydrophobic chains) in order to obtain polymeric micelles of PHEA-EDA-PS_{80}-PLA potentially useful to release drugs to the central nervous system. In this paper, the interaction and absorption of PHEA-EDA-PS_{80}-PLA micelles loaded with (*R*)-flurbiprofen with biomembrane models, represented by multilamellar or unilamellar vesicles made of dimyristoylphosphatidylcholine, are investigated by means of differential scanning calorimetry technique. (*R*)-Flurbiprofen is the single enantiomer of the racemate flurbiprofen; the capacity of this nonsteroidal anti-inflammatory drug to reduce risk of Alzheimer's disease has been recently reported. Drug release from the micelles to the lipid vesicles has been investigated in simulated physiological fluid, and it resulted to be affected by the biomembrane model.



KEYWORDS: polymeric micelles, polyaspartamide, polylactide, polysorbate, (*R*)-flurbiprofen, differential scanning calorimetry, biomembrane model

INTRODUCTION

Recent epidemiological studies prove that long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of Alzheimer's disease (AD) development;[1,2] in fact, a few NSAIDs can specifically reduce β-amyloid protein (Aβ) levels,[3,4] in particular the 42 amino acid form, Aβ42, implicated in the formation of aggregates (senile plaques and neurofibrillary tangles) in the parenchyma and cerebral blood vessels, that is a prevalent feature of AD.[5] In addition, AD is characterized by a severe chronic inflammatory response due to the accumulation of reactive microglia surrounding the plaques.[6] However, the application of NSAIDs for this purpose shows as a side effect the potential gastrointestinal toxicity related to cyclooxygenase (COX) inhibition that prevents its chronic use, especially in elderly persons. The screening of NSAID derivatives has led to identification of (*R*)-flurbiprofen (*R*-Flu) as a promising selective Aβ42 lowering agent that may avoid this complication.[4] It is the single enantiomer of the racemate NSAID flurbiprofen; this molecule does not inhibit cyclooxygenase activity and does not undergo stereoinversion in humans,[7,8] however, (*R*)-flurbiprofen seems to maintain some anti-inflammatory properties.[9] Like the racemate, (*R*)-flurbiprofen maintains the ability to lower Aβ42 in cell cultures.[4,10] However, delivery of drugs, such as *R*-Flu, to the brain is difficult because of the presence of the blood−brain barrier (BBB). Successful delivery can be achieved through the use of biocompatible nanocarriers.[11,12]

Amphiphilic copolymers for their ability to self-assemble as micelle-like aggregates,[13] with a suitable loading capacity, are of emerging interest for the delivery of water-insoluble drugs.[11,14] α,β-Poly[(*N*-hydroxyethyl)-DL-aspartamide] (PHEA) is a water-soluble synthetic polymer that lacks toxicity, antigenicity and immunogenicity;[15] it is suitable to obtain amphiphilic graft copolymers.[16] These copolymers can be obtained from PHEA-ethylenediamine (PHEA-EDA),[17,18] and functionalized with an aliphatic polyester, polylactide (PLA), in order to add hydrophobic

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side chains. Micelles targeting to specific areas can be achieved by attaching ligand molecules to the micelle surface; in particular, micelles that are coated with polysorbate 80 (PS_{80}) have the capability to transport the loaded drugs across the BBB.[19−21]

Therefore, the administration of *R*-Flu by means of a colloidal system, such as polymeric micelles based on PHEA-EDA-PS_{80}-PLA containing targeting moieties toward brain (PS_{80} residues), could potentially improve the efficacy of this drug and then allow results to be obtained that are different in comparison with the administration of the free drug. In this paper, the interaction and absorption of *R*-Flu loaded in PHEA-EDA-PS_{80}-PLA micelles with biomembrane models have been investigated. In particular, multilamellar vesicles (MLV) and unilamellar vesicles (LUV) made of dimyristoylphosphatidylcholine (DMPC) have been used as a biomembrane model. Biomembrane models including sphingolipids should better mimic brain cells. However, we decided to use only DMPC for two main reasons: (1) DMPC permits following the transfer of the drug by the delivery system to the biomembrane model at 37 °C, which is a temperature very close to the physiological temperature. Sphingolipids display a much higher transition temperature than glycerolipids, and their insertion in phospholipid bilayers increases the transition temperature of the system. (2) The calorimetric signal of lipid mixtures is too large, and, as a consequence, the effect of the drug or of the polymer on the MLV is difficult to be determined.

The release of NSAIDs from polymeric systems to multilamellar or unilamellar vesicles has been already monitored by differential scanning calorimetry (DSC).[22−24] DSC is a suitable technique to study the interaction of drugs and lipids and the transfer kinetics of an active molecule from a drug delivery system to a biomembrane model. The amount of *R*-Flu released from micelles and interacting with the lipid vesicles has been quantified by comparing the effects caused on the thermodynamic parameters of the vesicles with the effect obtained by the dispersion of increasing molar fractions of *R*-Flu in the lipid vesicles.

EXPERIMENTAL SECTION

Materials. All of the reagents used were of analytical grade, unless otherwise stated.

Polysorbate 80 (PS_{80}), *N*-hydroxysuccinimide sodium salt (NHS), bis(4-nitrophenyl)carbonate (PNPC), anhydrous *N*,*N*-dimethylacetamide (DMA), D_2O and DMSO-d_6 (isotopic purity 99.9%), were purchased from Sigma-Aldrich (Italy).

(*R*)-Flurbiprofen (*R*-Flu), ethylenediamine (EDA), diethylamine (DEA) and dimethyl sulfoxide (DMSO) were purchased from Fluka (Italy); RESOMER R 202 (D,L-polylactic acid, PLA: 8000 Da) was purchased from Bidachem-Boeringher Ingelheim (Italy).

Synthetic 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, purity 99%) was obtained from Genzyme (Switzerland).

α,β-Poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and purified according to a procedure reported elsewhere.[25]

The batch of PHEA used in the present study had a weight-average molecular weight ($\overline{M_W}$) of 42.6 kDa ($\overline{M_W M_n}$ = 1.7), determined by size-exclusion chromatography (SEC), using a SEC system equipped with a pump system, two Phenogel columns from Phenomenex (5 μm particle size, 10^3 and 10^4 Å of pore size) and a 410 differential refractometer (DRI) as a concentration detector (Waters, Mildford, MA). PEO/PEG as standards (range 232−932000 Da), DMF + 0.01 M LiBr as a mobile phase and a flow of 0.8 mL min^{-1}, operating at 50 °C (±0.1 °C) were used.

Synthesis of PHEA-EDA, PHEA-EDA-PS_{80} and PHEA-EDA-PS_{80}-PLA copolymers. Derivatization of PHEA with EDA to obtain PHEA-EDA copolymer and characterization were carried out according to a procedure described elsewhere.[17] The product, dissolved in bidistilled water and purified by extensive dialysis with Visking dialysis tubing, 12000−14000 cutoff, was recovered, after freeze-drying, with a yield of 95 wt %, based on the starting PHEA. The degree of derivatization in EDA (DD_{EDA}), determined by ^{1}H NMR spectroscopy in D_2O and calculated according to the method reported elsewhere, was 30.0 ± 0.5 mol %.

The weight-average molecular weight ($\overline{M_W}$) of PHEA-EDA copolymer, determined by SEC analysis, was found to be 51.3 kDa ($\overline{M_W M_n}$ = 1.8).

Derivatization of PHEA-EDA with PS_{80} to obtain PHEA-EDA-PS_{80} copolymer was performed as follows. Hydroxylic groups of PS_{80} were activated in anhydrous DMA solution with PNPC at 10 °C for 3 h. After this activation time, a PHEA-EDA solution was added and the final reaction mixture 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 $X = 1$ and $Y = 1$, where

$$X = \frac{\text{moles of PNPC}}{\text{moles of } PS_{80}}$$

$$Y = \frac{\text{moles of } PS_{80}}{\text{moles of amino pendant groups in PHEA-EDA}}$$

After this time, the reaction mixture was precipitated in diethyl ether (200 mL) and centrifuged for 15 min, at 11800 rpm and 4 °C. The product was recovered, washed several times with acetone, then dissolved in bidistilled water and purified by extensive dialysis with Visking dialysis tubing, 12000−14000 cutoff. Finally the solution was dried by freeze-drying. PHEA-EDA-PS_{80} was obtained with a yield of 85 wt %, based on the starting PHEA-EDA. The degree of derivatization in PS_{80} (DD_{PS80}), determined by ^{1}H NMR spectroscopy in D_2O, was found to be 1.2 ± 0.03 mol %. The $\overline{M_W}$ of PHEA-EDA-PS_{80} copolymer determined by SEC measurements was found equal to 56.4 kDa ($\overline{M_W M_n}$ = 1.8).

Derivatization of PHEA-EDA-PS_{80} with polylactic acid (PLA) to obtain the amphiphilic PHEA-EDA-PS_{80}-PLA was performed as follows. 27.5 mg mL^{-1} of PHEA-EDA-PS_{80} was dissolved in 2 mL of anhydrous DMSO at 40 °C, and then an appropriate amount of DEA, used as a catalyst, was added. NHS derivative of PLA (PLA-NHS) obtained as reported elsewhere[26] was added to PHEA-EDA-PS_{80} solution over 1 h. The reaction was carried out under argon at 40 °C for 4 h and then overnight at 25 °C. The employed amounts of PLA-NHS and DEA were calculated according to $X^1 = 0.035$ and $Y^1 = 1.2$, where

$$X^1 = \frac{\text{moles of PLA-NHS}}{\text{moles of amino pendant groups in PHEA-EDA-}PS_{80}}$$

$$Y^1 = \frac{\text{moles of EDA}}{\text{moles of amino pendant groups in PHEA-EDA-}PS_{80}}$$

After this time, the reaction mixture was precipitated in diethyl ether (200 mL) and centrifuged for 15 min at 11800 rpm and 4 °C. The product was recovered, washed several times with

acetone, and then dried under vacuum. PHEA-EDA-PS_{80}-PLA was obtained with a yield of 100% based on the initial polymer PHEA-EDA-PS_{80}. The degree of derivatization in PLA (DD_{PLA}), determined by ^{1}H NMR spectroscopy in DMSO-d_6, was found to be 0.54 ± 0.05 mol %. The weight-average molecular weight of PHEA-EDA-PS_{80}-PLA copolymer, determined by SEC analysis, was found to be 71.0 kDa ($\overline{M_W M_n}$ = 2.0).

Preparation of Micelles from PHEA-EDA-PS_{80}-PLA Copolymer. In a typical procedure, 50 mg of the PHEA-EDA-PS_{80}-PLA copolymer was dissolved in 2 mL of dimethyl sulfoxide (DMSO). Subsequently, the solution was transferred into a preswollen dialysis membrane (Spectra/POR; molecular weight cutoff 10000) and dialyzed against phosphate buffer solution (PBS) pH 7.4 in order to induce aggregation of the copolymer. The mixture solution was stirred and dialyzed for 2 days to remove most the DMSO. Afterward micelle dispersion was lyophilized.

Determination of Critical Aggregation Concentration (CAC) by Fluorescence Spectroscopy. The critical aggregation concentration (CAC) of PHEA-EDA-PS_{80}-PLA copolymer was determined by fluorescence measurements using pyrene as a probe. First, a stock solution of pyrene in acetone (6.0×10^{-2} M) was prepared, and then, to obtain the fluorescence spectrum, the pyrene solution was diluted with PBS pH 7.4 to a pyrene concentration of 12×10^{-7} M. The final pyrene solution was then distilled under vacuum at 60 °C for 2 h to remove acetone. The acetone-free pyrene solution was mixed with PHEA-EDA-PS_{80}-PLA dissolved in PBS pH 7.4 with concentrations ranging from 1×10^{-5} to 50 mg mL^{-1}, bringing the final concentration of pyrene to 6.0×10^{-7} M. The solutions were kept for 24 h under continuous stirring before the analysis, and then they were placed in quartz cuvettes and outgassed by bubbling with oxygen-free nitrogen for 5 min before recording the spectra.

The fluorescence spectra were recorded with a RF-5301PC spectrofluorometer (Shimadzu, Italy). The slit openings were 3 mm, the excitation wavelength was 333 nm and wavelengths of I_1 and I_3 appeared at 372−378 and 384−388 nm, respectively.

Determination of Micelle Mean Size. The mean diameter and width of distribution (polydispersity index, PDI) of the obtained micelles were determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.). The measurements were carried out at a fixed angle of 173° at a temperature of 25 °C by using PBS pH 7.4 as a suspending medium and a copolymer concentration equal to 5 mg mL^{-1}. The deconvolution of the measured correlation curve to an intensity size distribution was accomplished by using a nonnegative least-squares algorithm.

Evaluation of Stability of Micelles. To test the stability of PHEA-EDA-PS_{80}-PLA micelles in conditions mimicking the physiological ones, micelles were dispersed in PBS pH 7.4 and incubated for 48 h at 37 °C under continuous stirring. At scheduled times, micelle solutions were analyzed by photon correlation spectroscopy to evaluate variations in their mean size and PDI.

Preparation of *R*-Flu-Loaded PHEA-EDA-PS_{80}-PLA Micelles. *R*-Flu-loaded PHEA-EDA-PS_{80}-PLA micelles were prepared by closely mixing, using pestle and mortar, appropriate amounts of PHEA-EDA-PS_{80}-PLA and *R*-Flu, using a published procedure.[27] In particular, a copolymer/drug weight ratio equal to 1/1 was used; then, 1 mL of ethanol was added to dissolve *R*-Flu and aliquots of 500 μL of phosphate buffer solution pH 7.4 were added until a volume of 8 mL was reached. The obtained dispersion was stirred for 1 h, by using an Ultraturrax for 5 min, submitted to ultrasound for 1 h and then centrifuged at 11,800 rpm at 25 °C for 20 min. The supernatant was filtered on cellulose acetate filter (0.2 μm), transferred into a preswollen dialysis membrane (Spectra/POR; molecular weight cutoff 10000) and dialyzed against PBS pH 7.4. Afterward drug loaded micelle dispersion was lyophilized.

To evaluate the amount of *R*-Flu loaded into PHEA-EDA-PS_{80}-PLA micelles, a HPLC analysis was performed by using a μBondapak column (5 μm, 250 × 46 mm i.d., Waters); the mobile phase was a mixture methanol and phosphoric acid (1% v/v) 80:20 v/v with a flow rate of 1.0 mL/min, reading at λ = 247 nm.

5 mg of *R*-Flu-loaded PHEA-EDA-PS_{80}-PLA micelles was dissolved in 10 mL of bidistilled water, filtered with a 0.2 μm cellulose membrane filter and analyzed by HPLC. *R*-Flu amount loaded into PHEA-EDA-PS_{80}-PLA micelles was determined by means of a calibration curve obtained by using standard solutions of *R*-Flu in ethanol in the range of concentrations 5−100 μg/mL (y = 57410x, R^2 = 0.9958). Results were expressed as the weight percent ratio between the drug loaded and the dried system (copolymer + drug).

Solubility Studies of *R*-Flu. Solubility of *R*-Flu was determined by shaking an excess of drug in bidistilled water at 25 °C. The suspension was sonicated for 10 min, centrifuged at 8500 rpm for 7 min, and filtered on a 0.45 μm cellulose membrane. The *R*-Flu amount in saturated solution was evaluated by HPLC analysis.

Water solubility of *R*-Flu was also evaluated in the presence of PHEA-EDA-PS_{80}-PLA micelles as follows: 50 mg of *R*-Flu was closely mixed with 50 mg of copolymer, and 5 mL of bidistilled water was added. The obtained suspension was sonicated for 10 min, centrifuged at 8500 rpm for 7 min, filtered on a cellulose acetate membrane (0.2 μm) and analyzed by HPLC.

Liposome Preparation. Stock solutions of DMPC and *R*-Flu were prepared in chloroform−methanol (1:1 v:v), and then appropriate aliquots were mixed in glass flasks to obtain the same amount of DMPC (0.010325 mmol) and increasing molar fraction (X) (0.0, 0.015, 0.03, 0.045, 0.06, 0.09, 0.12) of *R*-Flu with respect to the DMPC. The solvents were removed under a nitrogen flow, and the resulting films were freeze-dried under vacuum to remove the residual solvents. Lipid films were suspended with 168 μL of TRIS 50 mM, pH 7.4, and the multilamellar vesicles (MLV) were prepared by heating to 37 °C (temperature above the gel−liquid crystalline phase transition) for 1 min and vortexing for 1 min, for three times. The samples were kept for 1 h in a water bath at 37 °C to homogenize the liposomes and to reach a complete distribution of *R*-Flu between lipid and aqueous phases.

To obtain large unilamellar vesicles (LUV) (for X 0.0 and 0.09), MLV were repetitively (19 times) passed under moderate pressure at a temperature at least 5 °C above the T_m through polycarbonate membranes (pore diameter 100 nm) in an extruder system LiposoFast Basic, Avestin Inc.[28,29] The membrane pores are almost cylindrical, and vesicles are reduced in size and lamellarity during the passage through the pores.

The vesicle size of both MLV and LUV was checked by standard dynamic light scattering measurements using a Zetasizer Nano SZ90 (Malvern Instruments, Malvern, U.K.). MLV showed a mean diameter of 1517.5 nm ± 82.73 nm, with a polydispersity index (PDI) = 0.03. LUV showed a mean diameter of 120.2 nm ± 6.01 nm, with a PDI = 0.15.

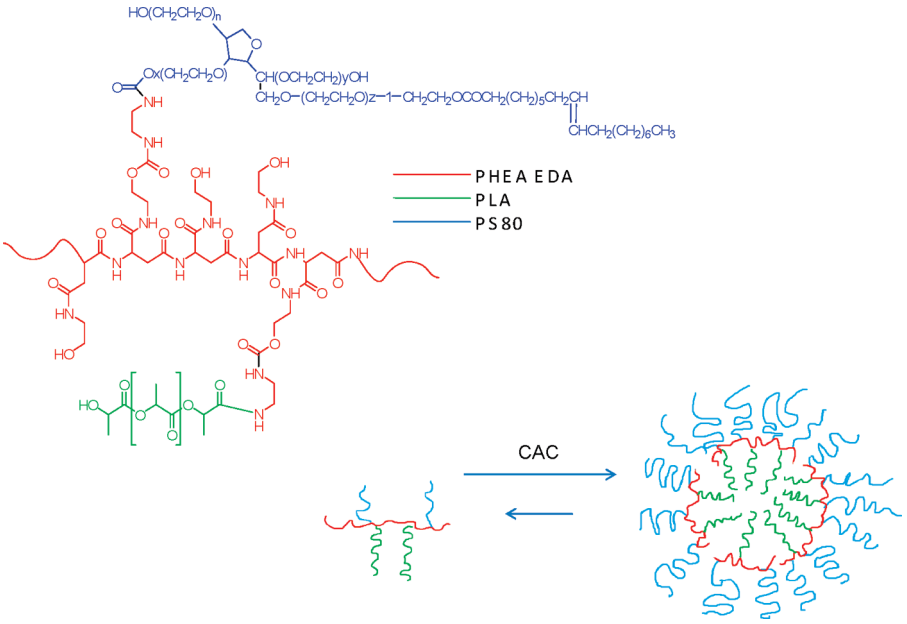

Figure 1. Chemical structure of PHEA-EDA-PS_{80}-PLA copolymer and schematic formation of micelles in PBS pH 7.4.

DSC Analysis of *R*-Flu/Vesicles Interaction. A Mettler Toledo STAR^e system equipped with a DSC-822^e calorimetric cell and a Mettler TA-STAR^e software was used. The sensitivity was automatically chosen as the maximum possible by the calorimetric system, and the reference pan was filled with TRIS solution. The calorimetric system was calibrated, in transition temperature and enthalpy changes, by using indium, stearic acid and cyclohexane by following the procedure of the DSC 822 Mettler TA STAR^e instrument.

120 μL of MLV or LUV with or without *R*-Flu was transferred into a 160 μL aluminum DSC pan, which was sealed, and submitted to calorimetric analysis as follows: (i) a heating scan between 5 and 37 at 2 °C/min; (ii) a cooling scan between 37 and 5 at 4 °C/min; for at least three times to check the results reproducibility. After the DSC analysis, aliquots of all samples were extracted from the calorimetric aluminum pans and used to determine, by the phosphorus assay,[30] the exact amount of phospholipids present in each sample.

Kinetic Experiments. These experiments were carried out to follow the transfer and the absorption by biomembrane models of *R*-Flu as a free drug or released by the micelles. An exact amount of free *R*-Flu or *R*-Flu loaded micelles in order to obtain a 0.09 molar fraction of drug with respect to the phospholipid was weighed in the bottom of the DSC pan, and 120 μL of MLV or LUV was added. The aluminum pan was hermetically closed and submitted to the following calorimetric analysis: (i) a heating scan between 5 and 37 °C at the rate of 2 °C/min; (ii) an isothermal period (1 h) at 37 °C; and (iii) a cooling scan between 37 and 5 °C at the rate of 4 °C/min. This procedure was repeated eight times to follow the variations in the calorimetric curves, which indicate that an interaction between the tested compounds and the DMPC vesicles occurs. Furthermore, to be sure that the variation on DMPC vesicles calorimetric curves is due only to the released drug, an exact amount of unloaded micelles (the same employed in the *R*-Flu release experiment) was left in contact with 120 μL of MLV or LUV at the same increasing incubation times of the experiments carried out with *R*-Flu or *R*-Flu loaded micelles.

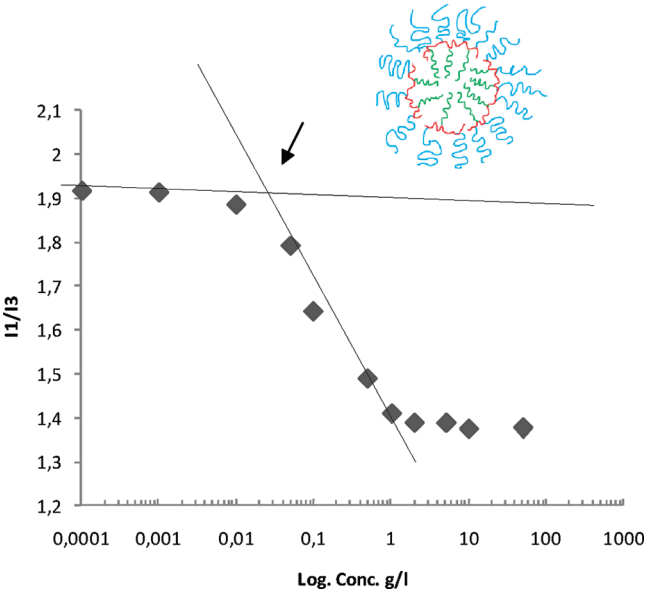

Figure 2. Plot of the intensity ratio I_1/I_3 as a function of PHEA-EDA-PS_{80}-PLA copolymer concentration in PBS pH 7.4. λ_{exc} = 333 nm. The arrow shows the intersection between the tangent to the curve at the inflection point with the horizontal tangent of the plateau line. The critical aggregation concentration (CAC) corresponds to the value found, in the *X* axis, at this intersection point.

RESULTS AND DISCUSSION

Polymeric micelles as delivery systems for *R*-Flu were obtained by using PHEA-EDA-PS_{80}-PLA copolymer. In particular, starting polymer was PHEA, a biocompatible water-soluble synthetic polymer, whose use in various biomedical and pharmaceutical fields has been reported in previous papers.[31−33] PHEA has been modified by reaction with ethylendiamine (EDA) in order to introduce amino groups in its side chains more reactive than hydroxyl groups of native polymer, thus obtaining PHEA-EDA

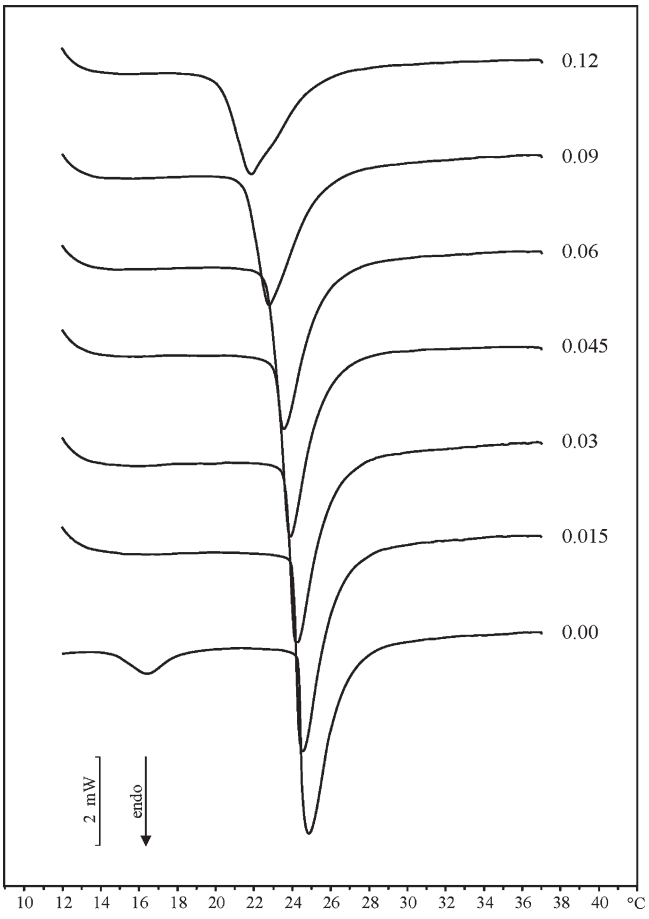

Figure 3. Calorimetric curves, in heating mode, of MLV prepared in the presence of increasing molar fractions of *R*-Flu at pH 7.4).

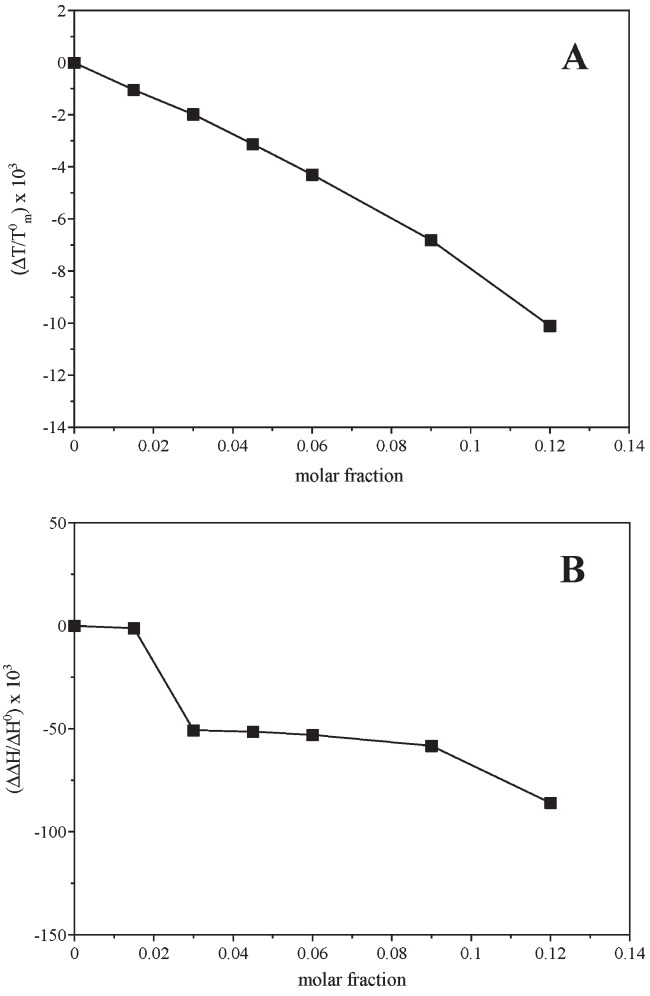

Figure 4. Transition temperature variation (A) and enthalpy change (B) reported as $\Delta T/T°_m$ and $\Delta\Delta H/\Delta H°$, respectively, as a function of *R*-Flu molar fraction. $\Delta T = T_m - T°_m$, where T_m is the transition temperature of *R*-Flu loaded MLV and $T°_m$ is the transition temperature of unloaded MLV. $\Delta\Delta H = \Delta H - \Delta H°$; where ΔH is the enthalpy change of *R*-Flu loaded MLV and $\Delta H°$ is the enthalpy change of unloaded MLV.

copolymer that was linked to PS_{80} and PLA. This aliphatic polyester, approved by FDA and used for various biomedical applications due to its biocompatibility and biodegradability, has been chosen to modify the affinity of the polyaspartamide derivative (PHEA-EDA) toward the aqueous medium with the aim to obtain amphiphilic copolymer able to aggregate as polymeric micelles in this medium. On the other hand, the introduction of PS_{80} moieties in polymeric backbone was carried out to obtained systems potentially useful for drug targeting to the central nervous system (CNS), as reported in the literature.[19−21]

The chemical structure of PHEA-EDA-PS_{80}-PLA copolymer is reported in Figure 1 together with the schematic formation of micelles. The dialysis method (see Experimental Section) was chosen to prepare micelles through self-aggregation in PSB pH 7.4. In particular, during the DMSO removal caused by the dialysis process, the PLA chains form the hydrophobic core whereas PS_{80} residues and PHEA-EDA chains form the hydrophilic shell.

In order to evaluate the critical aggregation concentration (CAC) for PHEA-EDA-PS_{80}-PLA copolymer in phosphate buffer solution pH 7.4, a fluorescence study using pyrene as a probe was carried out. In particular, pyrene is preferentially transferred from the aqueous medium to the hydrophobic inner core of micelles; this causes a variation in the pyrene intensity ratio I_1/I_3.

In Figure 2, the plot of I_1/I_3 versus the logarithm of the copolymer concentration is reported. CAC values can be calculated by the intersection of the tangent to the curve at the inflection point with the horizontal tangent of the plateau line. From this plot, a CAC value of 0.025 g/L was found.

To confirm the formation of colloidal structures in PBS pH 7.4, PHEA-EDA-PS_{80}-PLA micelles were also characterized in terms of mean size and polydispersity index (PDI). PHEA-EDA-PS_{80}-PLA micelles show a mean diameter of 25.6 nm ± 1.2 nm, suitable for all administration routes, also the intravenous one, and a low polydispersity index (PDI = 0.31).

No significant change in mean size and PDI of micelles has been found all through 48 h in PBS pH 7.4 thus confirming their stability in this medium; in fact the mean diameter was 24.9 ± 0.8 nm with a PDI = 0.28.

PHEA-EDA-PS_{80}-PLA micelles containing *R*-Flu were prepared with the aim to increase drug solubility and to promote, potentially, its release to the brain. In fact, as reported in the Introduction, *R*-Flu is a nonsteroidal anti-inflammatory drug which has the ability to selectively lower amyloid peptide production by modulation of enzymes such as gamma-secretase, responsible for the peptide formation from its precursor. This drug,

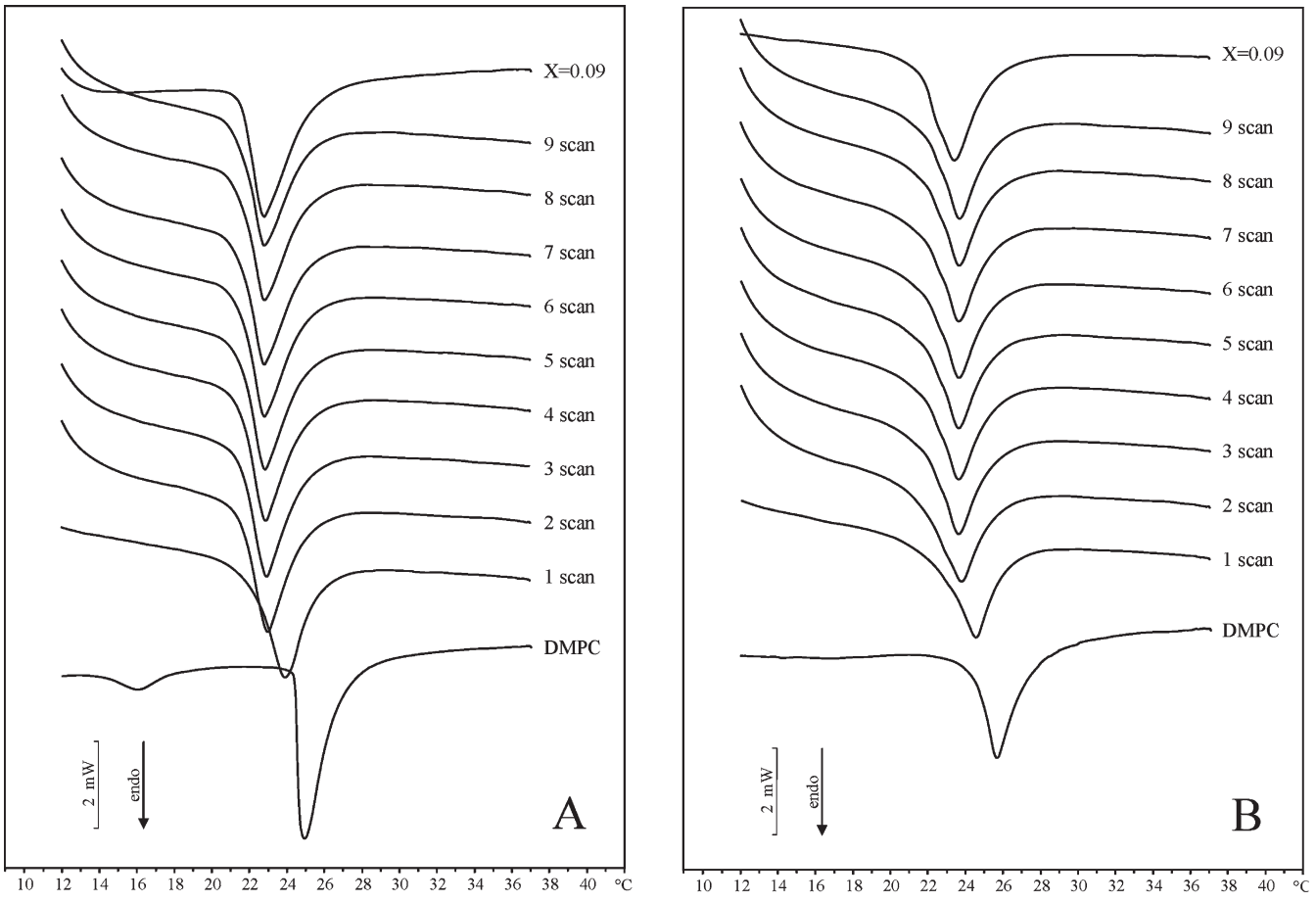

Figure 5. Calorimetric curves, in heating mode, of MLV (A) or LUV (B) left in contact with *R*-Flu, at pH 7.4.

however, after administration, binds to plasmatic proteins (>99%) and shows a short half-life. The incorporation of this drug into nanosystems targeted to CNS (due to the presence of PS_{80}) could increase its solubility, protect it from the binding with plasmatic proteins, prolong its half-life and promote its release in the target site.

The amount percentage of *R*-Flu loaded in PHEA-EDA-PS_{80}-PLA micelles, determined by HPLC measurements (see Experimental Section), resulted to be 4.0% w/w, showing a good ability of these micelles to entrap lipophilic molecules, such as *R*-Flu, into their hydrophobic inner core. Solubility studies of *R*-Flu in the absence and in the presence of PHEA-EDA-PS_{80}-PLA micelles have shown that investigated micelles are able to increase water solubility of this drug from 8 mg/mL to about 150 mg/mL.

To ascertain that *R*-Flu interacts with the vesicles, MLV of DMPC in the presence of increasing molar fraction (X) of *R*-Flu were prepared and analyzed by DSC; the obtained calorimetric curves are reported in Figure 3. The interaction of *R*-Flu with the MLV was evaluated by the modifications in the MLV calorimetric curve. In the temperature range used in our study (5−37 °C), MLV of pure DMPC, when submitted to heating, exhibit a small calorimetric peak (pretransition peak), at about 16 °C, related to the tilt of hydrophobic chains and a main calorimetric peak, at about 24.8 °C, related to the phase transition from the ordered gel state (L_β) to the liquid-crystalline phase (L_α). Variations of the thermodynamic parameters, transition temperature and enthalpy changes, of the phase transitions are caused by the presence of molecules dissolved in the lipid bilayer and are related to the amount of substance interacting with the bilayer.[34,35] *R*-Flu interacts with the biomembrane model, in fact, the pretransition peak already at the lower molar fraction disappears; the main transition peak shifts toward lower temperatures moreover, and the peak broadening is clearly visible. The drug, at 0.12 molar fraction, is not homogeneously dispersed in the phospholipid bilayers as it is suggested by the presence of a phase separation and the formation of poor and rich *R*-Flu regions.[36]

In Figure 4A,B the transition temperature variation and the enthalpy change are reported as $\Delta T/T^\circ_m$ and $\Delta\Delta H/\Delta H^\circ$, respectively ($\Delta T = T_m - T^\circ_m$, where T_m is the transition temperature of *R*-Flu containing MLV and T°_m is the transition temperature of pure DMPC MLV; $\Delta\Delta H = \Delta H - \Delta H^\circ$, where ΔH is the enthalpy change of *R*-Flu containing MLV and ΔH° is the enthalpy change of pure DMPC MLV), as a function of *R*-Flu molar fraction. A decrease in the transition temperature and in the enthalpy change occurs, is dependent on the amount of drug present in the MLV dispersion, and can be explained as a fluidifying effect caused by the intercalation of *R*-Flu in the ordered lipid structure.[37]

The analysis of these results allowed the choice of a molar fraction of 0.09 for the release experiments, as at this molar fraction the drug produces a strong effect on the MLV even if the peak shape remains well-defined. Then, even to study the interaction between *R*-Flu and LUV, we prepared and submitted

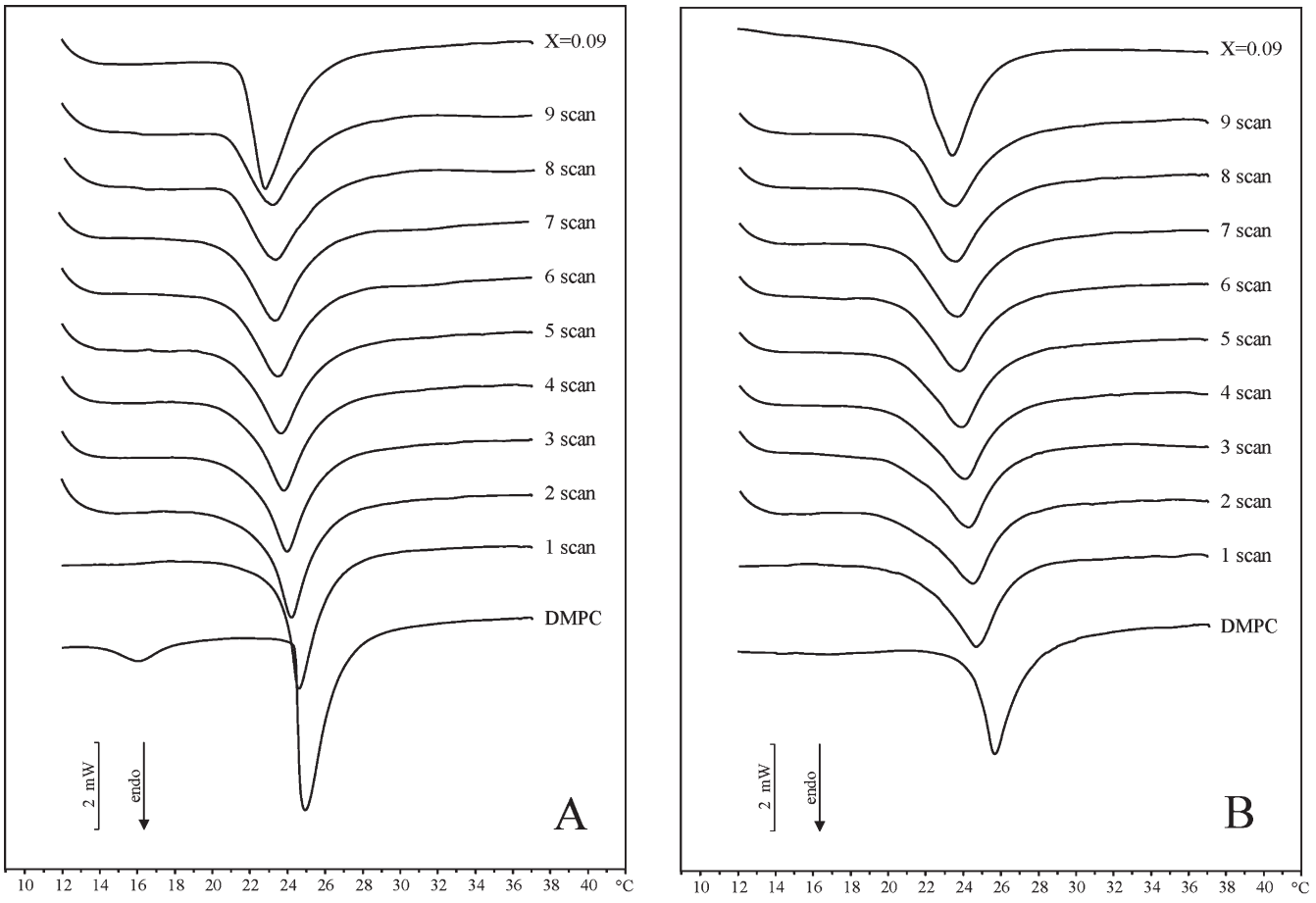

Figure 6. Calorimetric curves, in heating mode, of MLV (A) or LUV (B) left in contact with *R*-Flu loaded micelles, at pH 7.4.

to DSC analysis LUV in the absence and in the presence of drug at 0.09 molar fraction. *R*-Flu causes the peak of LUV to decrease and move toward lower temperature (data not shown).

The release of *R*-Flu from the micelles was detected following the variation in the calorimetric curves of DMPC liposomes due to the interaction of the drug with the phospholipid. 120 μL of DMPC MLV or LUV was left in contact with *R*-Flu or *R*-Flu loaded PHEA-EDA-PS_{80}-PLA micelles in order to obtain a 0.09 molar fraction of *R*-Flu with respect to the DMPC, for increasing time of incubation.

A fixed amount of PHEA-EDA-PS_{80}-PLA micelles (the same amount employed in the experiments with *R*-Flu loaded micelles) was incubated with MLV or LUV to ascertain that the variations of the calorimetric curves in the experiments carried out with *R*-Flu loaded micelles were due to the drug released and not to the copolymer.

LUV, possessing just one bilayer, expose to the environment the maximum surface able to absorb the drug and, then, permit evaluation of the ability of the structured phospholipid to catch the drug (as a free form or released from the micelles); in this way only the release of the drug from the micelles and its transfer through the aqueous medium are considered. MLV, in addition, permit getting an indication on the transfer of the drug from the outer to the inner bilayers; in fact, the drug absorbed by the outer bilayer can pass to the inner bilayers, by a flip flop mechanism.

The calorimetric curves of the experiments carried out with micelles alone (not shown) do not indicate any variation with respect to the calorimetric curves of liposomes all through the incubation time. For this reason, in the experiments carried out with drug loaded micelles, the effects on the thermotropic behavior of MLV and LUV have to be attributed only to the presence of *R*-Flu in the bilayers.

As far as the free *R*-Flu is concerned, the calorimetric curves (Figure 5A,B) are compared with that of MLV and LUV and that of MLV and LUV prepared in the presence of a 0.09 molar fraction of drug (X = 0.09), obtained as described in Liposome Preparation, which represents the maximum possible interaction between *R*-Flu and vesicles. This is the value that should be reached if a complete release, transfer and absorption of the drug by the vesicles happened. With regard to MLV, *R*-Flu (Figure 5A) causes the pretransition peak to disappear and the calorimetric peak to broaden and shift toward lower temperature as the incubation time increases. With regard to LUV (Figure 5B), a gradual shift of the main peak toward lower temperature as the incubation time increases is observed.

Figure 6A,B shows the calorimetric curves of *R*-Flu loaded micelles left in contact with MLV and LUV. In the calorimetric curves of MLV, the pretransition peak disappears as soon as after the contact, and the main peak shifts toward lower temperature and widely broadens. The calorimetric curves of LUV show, as the incubation time increases, the shift toward lower temperature and a broadening of the peak.

In Figure 7 the transition temperature variations, as $\Delta T/T°_m$, of the calorimetric curves of MLV or LUV left in contact with a

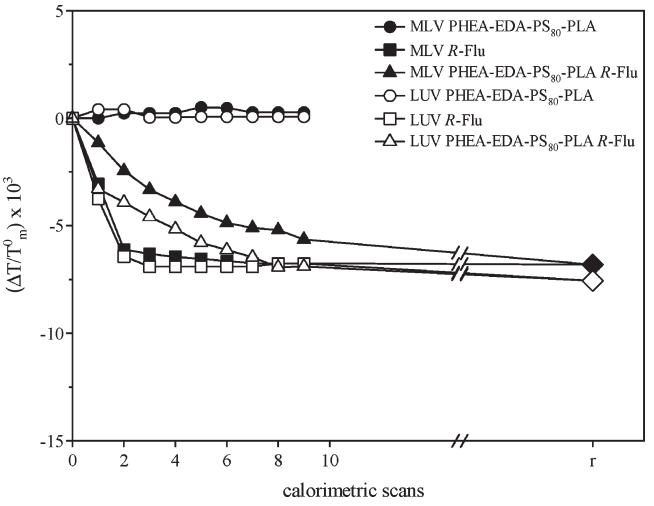

Figure 7. Transition temperature variation of MLV or LUV left in contact with unloaded micelles, *R*-Flu and *R*-Flu loaded micelles, as a function of the calorimetric scans, at pH 7.4. The *r* value represents the complete transfer of the drug to the vesicles.

fixed amount of PHEA-EDA-PS_{80}-PLA micelles, *R*-Flu and *R*-Flu loaded PHEA-EDA-PS_{80}-PLA micelles, as a function of the calorimetric scans, are reported. The *r* value is the transition temperature variation of MLV or LUV prepared in the presence of a 0.09 molar fraction of *R*-Flu (maximum interaction between drug and vesicles). The polymeric micelles do not cause transition temperature variation. *R*-Flu alone causes a great decrease in the transition temperature, both of MLV and of LUV, within the first two scans, and afterward the variation remains unchanged and the value *r* is reached. In the experiments carried out with loaded micelles the temperature decrease is gradual, both for MLV and for LUV, and the *r* value is almost reached.

The results indicate that the absorption by MLV and LUV is very fast for *R*-Flu, alone indicating that the drug dissolves in the medium, reaches the vesicle surface and is absorbed by the bilayers; in addition, the almost overlapping of the data of MLV and LUV indicates that *R*-Flu, besides being taken up by the vesicles (MLV and LUV), transfers from the outer layer to the inner layers of the MLV. When *R*-Flu is loaded in the micelles, the absorption, even if complete, is more gradual as the drug has to diffuse through the carrier. On the basis of these results, it can be hypothesized that the micelles could give rise to a gradual release of the drug and consequently to a gradual entry into the cells where it modifies the gamma-secretase with the consequent lowering of the Aβ42. In particular, the polymer could affect the vesicles, increasing their surface area and, then, favoring the entry of the drug.

CONCLUSION

PHEA-EDA-PS_{80}-PLA micelles were prepared and loaded with (*R*)-flurbiprofen. The ability of micelles to release the drug to lipidic vesicles was studied by differential scanning calorimetry. The release was evaluated by the modification in the calorimetric curves of MLV or LUV (chosen as biomembrane models) due to the absorption of the drug. The experimental results showed that PHEA-EDA-PS_{80}-PLA micelles allow a gradual release of (*R*)-flurbiprofen which could permit a prolonged activity of the drug into the cells to be obtained. Moreover, the results indicate that *R*-Flu transfers from the outer layer to the inner layers of the MLV.

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