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Influence of functionalization on interaction and drug release from α,β-polyaspartylhydrazide derivatives to a biomembrane model: evaluation by differential scanning calorimetry technique

Francesco Castelli [a], Chiara Messina [a], Elisa Martinetti [a], Mariano Licciardi [b], Emanuela Fabiola Craparo [b], Giovanna Pitarresi [b,*]

[a] *Dipartimento di Scienze Chimiche, Università di Catania, Viale A. Doria, 6-95125 Catania, Italy*
[b] *Dipartimento di Chimica e Tecnologie Farmaceutiche, Università di Palermo, Via Archirafi, 32-90123 Palermo, Italy*

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Abstract

A comparative study on the ability of various polymers to interact with a biomembrane model was carried out by differential scanning calorimetry (DSC). The investigated samples were a water soluble polymer, the α,β-polyaspartylhydrazide (PAHy) and its derivatives containing polyethylene glycol (PEG_{2000}) (sample PAHy–PEG_{2000}), or hexadecylamine (C_{16}) (sample PAHy–C_{16}) or both compounds (sample PAHy–PEG_{2000}–C_{16}). Some samples are able to arrange themselves as micellar structures and to interact potentially with the membrane surface so as to favor the release of the drug near the target membrane and consequently to improve drug adsorption processes. First, the interaction of all polymers with a biomembrane model made of multilamellar vesicles of mixed (80:20, mol:mol) dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidic acid (DMPA), has been studied (pH 7.4), then the interaction of Ketoprofen (KPF) as free drug or released from PAHy–C_{16} micelles with the same biomembrane model has been investigated. Differential scanning calorimetry appears to be a suitable technique to follow the interaction of all prepared samples as well as the transfer kinetics of a drug from PAHy–C_{16} micelles to a biomembrane model. The drug release kinetics, from PAHy–C_{16} micelles, was compared with the transfer of free drug (after dissolution from a solid form) by evaluating their effects on the thermotropic behavior of DMPC/DMPA multilamellar vesicles.

Keywords: Polyaspartylhydrazide; Polymeric micelles; Dimyristoylphosphatidylcholine; Dimyristoylphosphatidic acid; Differential scanning calorimetry; Drug release

1. Introduction

Functionalization of a biocompatible polymer, characterized by a remarkable water solubility with hydrophobic groups, modifies its physicochemical properties and it gives the opportunity to arrange oneself as supramolecular structures called "polymeric micelles" having, in an aqueous medium, an interior hydrophobic compartment and a hydrophilic external shell. These polymeric systems have received increased attention for their applications in pharmaceutical field [1,2]. In fact polymeric micelles, due to their peculiar structure, are able to increase the water solubility and bioavailability of lipophilic substances, such as drugs or nutrients, to reduce toxic and adverse effects and to enhance permeability across physiological membranes. In particular, the use of hydrophilic macromolecular chains, e.g. polyethylene glycol (PEG), to build polymeric surfactants, allows to obtain micelles able to escape the capture by the reticuloendothelial system thus prolonging their blood circulation [3]. Besides micelles may be targeted to a specific site by chemical attachment of targeting moiety to their surface [4] or by introducing temperature or pH sensitive chemical functions. In all cases, drug molecules can be loaded into polymeric micelles either via chemical conjugation or physical entrapment.

In this context our study is aimed at the preparation and characterization of new polymers based on α,β-polyaspartylhydrazide (PAHy) containing hydrophilic

* Corresponding author. Tel.: +39-091-6236154; fax: +39-091-6236150.
E-mail address: giopitar@unipa.it (G. Pitarresi).

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and/or hydrophobic groups so to modulate the ability to form micelles as well as to interact with biological membranes.

PAHy is a synthetic polymer that satisfies some fundamental requirements like water solubility, absence of toxical, antigenic and teratogenic effects and it has been already proposed as a plasma substitute, drug carrier and starting material to obtain chemical hydrogels [5–7]. PAHy derivatives prepared in this work, contain hexadecylamine (C_{16}) and/or polyethylene glycol (PEG_{2000}) groups. The presence of C_{16} in the backbone could allow an arrangement as polymeric micelles while, the presence of PEG_{2000} should balance the increase in the hydrophobic feature due to the introduction of alkylic moiety (C_{16}) and it allows to obtain polymeric micelles having a potential long permanence in blood circulation. Obviously the application of PAHy derivatives in a living organism, requires some features. These include the tolerance of these materials put in contact with tissues, organs and blood, as well as a suitable ability to interact with biological membranes.

For this reason, we have evaluated the interaction of PAHy derivatives (PAHy–PEG_{2000}, PAHy–C_{16} and PAHy–PEG_{2000}–C_{16}) with mixed dimyristoylphosphatidylcholine (DMPC)–dimyristoylphosphatidic acid (DMPA) (80:20, mol:mol) liposomes, chosen as biomembrane models using differential scanning calorimetry (DSC). The use of mixture DMPC/DMPA allows to obtain a model membrane having a negative charge like natural cellular membranes. In addition, in order to evaluate the potential use of PAHy–C_{16} micelles as drug carriers, Ketoprofen (KPF), a molecule practically insoluble in water, has been chosen as a model drug that has been physically entrapped into these micelles and its release to the biomembrane model has been also investigated.

Ketoprofen is a nonsteroidal anti-inflammatory drug with well established analgesic and antipyretic properties. It is widely used for the treatment of rheumatic disorders, arthritis and to moderate pains but it requires frequent administrations [8,9]. Oral therapy of Ketoprofen is very effective but clinical use is often limited because of adverse effects such as irritation and ulceration of the gastrointestinal tract. The drug entrapment in polymeric micelles should represent a good approach to obtain the release minimizing dangerous side effects.

Since it is known that polymers alone or together with drugs can interact with lipid vesicles by causing a T_m variation (i.e. the temperature of gel-to-liquid crystalline phase transition characteristic of a phospholipidic bilayer), we have studied the polymer–liposomes interaction and the drug release from polymeric micelles through DSC technique. In particular, these molecules, interacting with the lipidic sea, act as impurities destabilizing the lipidic ordered structure and shifting the T_m towards lower values with respect to the lipid alone [10–13]. According to Vant'Hoff model, the validity of this model application has been verified for different classes of compounds [14].

The suitability of DSC technique to follow the transfer of a drug molecule from various sustained release devices to a site mimicking a biological membrane has been already reported [15–17]. This technique appears to be a promising approach to study the drug release in like "in vivo" studies respect to the classical "in vitro" dissolution test.

2. Experimental

2.1. Materials

Hexadecylamine (C_{16}) was purchased from Aldrich (Germany). *O*-(2-aminoethyl)-*O*′-methylpolyethylene glycol 2000 (PEG_{2000}) ($\leq$0.4 mmol NH_2/g) was purchased from Fluka (Italy). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid sodium salt were obtained from Genzyme Pharmaceuticals (Switzerland). The lipid solution was chromatographically pure as assessed by two-dimensional thin-layer chromatography (TLC). Lipid concentration was determined by phosphorous analysis [18]. Ketoprofen (2-(3-benzoylphenyl)propionic acid) was provided by Sigma (USA).

Polysuccinimide (PSI) was prepared in a large yield by polycondensation of DL-aspartic acid according to a method reported in literature [19].

α,β-Polyaspartylhydrazide (PAHy) was prepared by reaction of a polysuccinimide (PSI), obtained by thermal polycondensation of DL-aspartic acid, with hydrazine in DMF solution and purified as reported elsewhere [5]. Analytical and spectral data (FT-IR and ^{1}H NMR) were in agreement with the literature values [5]. PAHy weight-average molecular weight was 23 500 g/mol (M_w/M_n = 1.78) as determined by multi-angle light scattering (MALS) [20].

2.2. Polymer synthesis

2.2.1. Synthesis of PSI derivatives

The synthesis of PSI–PEG_{2000} and PSI–PEG_{2000}–C_{16} derivatives was reported elsewhere [21]. For the synthesis of PSI–C_{16} derivative, a solution of hexadecylamine (C_{16}) in DMF (0.75 g/5 ml) was added dropwise at 60 °C to a solution of PSI in DMF (0.4 g/5 ml). The reaction mixture was maintained at 60 °C for 7 h under argon and continuous stirring, afterwards it has been precipitated with ethyl ether, washed several times with ethyl ether and finally dried under vacuum for several hours.

2.2.2. Synthesis of PAHy derivatives

For the synthesis of PAHy derivatives (i.e. PAHy–PEG_{2000}, PAHy–C_{16} and PAHy–PEG_{2000}–C_{16}), 1.24 ml of hydrazine hydrate were added dropwise to DMF solutions of PSI–PEG_{2000}, PSI–C_{16} or PSI–PEG_{2000}–C_{16} (1 g/15 ml) maintaining the reaction under stirring for 4 h and with a temperature between 22 and 26 °C. Each obtained sample was precipitated with ethyl ether, washed several times with

acetone and finally dried under vacuum. Then, each sample was dissolved in water and purified by exhaustive dialysis running distilled water using Visking dialysis tubing with a molecular cut off 12 000–14 000 g/mol. Each solution was lyophilized and each purified product, obtained with a 95% yield (based on the starting PSI), was characterized by FT-IR spectrophotometry (using a Perkin-Elmer 1720 Fourier transform spectrophotometer) and ^{1}H NMR (using a Bruker AC-250 instrument operating at 250.13 MHz) analysis. The spectral data of PAHy derivatives were in agreement with the attributed structures.

IR spectra of PAHy–PEG_{2000} (KBr) showed bands at 3300, 3210 and 3055 cm^{-1} (–NH_2, –NH–), 1657 cm^{-1} (amide I), 1542 cm^{-1} (amide II), 1244 cm^{-1} (amide III) and 644 cm^{-1} (amide IV) belonging to PAHy and 953 cm^{-1} (stretching of C–O ether of PEG).

^{1}H NMR of PAHy–PEG_{2000} (D_2O): δ 2.68 (m, 2H, –CO–CH–**C**$\mathbf{H}_2$–CO–NH–), 3.64 (s, 178H, –CH_2–**C**$\mathbf{H}_2$–O–), 4.64 (m, 1H, –NH–**C**$\mathbf{H}$(CO)CH_2).

IR spectra of PAHy–C_{16} (KBr) showed bands at 3300, 3210 and 3055 cm^{-1} (–NH_2, –NH–), 2985 and 2854 cm^{-1} (C–H stretching of C_{16} chains), 1657 cm^{-1} (amide I), 1542 cm^{-1} (amide II), 1244 cm^{-1} (amide III) and 644 cm^{-1} (amide IV) belonging to PAHy.

^{1}H NMR of PAHy–C_{16} (D_2O): δ 0.67 (t, 3H, –CH_2–**C**$\mathbf{H}_3$), 1.20 (m, 28H, –CH_2–**C**$\mathbf{H}_2$–CH_2), 2.69 (m, 2H, –CO–CH–**C**$\mathbf{H}_2$–CO–NH–), 4.64 (m, 1H, –NH–**C**$\mathbf{H}$(CO)CH_2). IR spectra of PAHy–PEG_{2000}–C_{16} (KBr) showed bands at 3300, 3210 and 3055 cm^{-1} (–NH_2, –NH–), 2985 and 2854 cm^{-1} (C–H stretching of C_{16} chains), 1657 cm^{-1} (amide I), 1542 cm^{-1} (amide II), 1244 cm^{-1} (amide III) and 644 cm^{-1} (amide IV) belonging to PAHy and 953 cm^{-1} (stretching of C–O ether of PEG).

^{1}H NMR of PAHy–PEG_{2000}–C_{16} (D_2O): δ 0.79 (t, 3H, –CH_2–**C**$\mathbf{H}_3$), 1.19 (m, 28H, –CH_2–**C**$\mathbf{H}_2$–CH_2), 2.68 (m, 2H, –CO–CH–**C**$\mathbf{H}_2$–CO–NH–), 3.64 (s, 178H, –CH_2–**C**$\mathbf{H}_2$–O), 4.67 (m, 1H, –NH–**C**$\mathbf{H}$(CO)CH_2).

The degree of derivatization (DD) of PAHy–PEG_{2000} derivative was calculated by ^{1}H NMR by comparing the integral of the peak corresponding to protons at 3.64δ assigned to –(CH_2–**C**$\mathbf{H}_2$–O)$_n$–, belonging to PEG with the integral of the peak related to protons at 2.68δ assigned to –CO–CH–**C**$\mathbf{H}_2$–CO–NH– belonging to PAHy. The derivatization degree was expressed as DD = (polyethylene glycol/polymer repeating unit) × 100 (mol). It was found for PAHy–PEG_{2000} a DD value of 1.4 mol%.

Similarly, the degree of derivatization of PAHy–C_{16} was calculated by ^{1}H NMR by comparing the integral of the peak corresponding to protons at 0.67δ assigned to –CH_2–**C**$\mathbf{H}_3$ (or the integral of peak related to protons at 1.20δ assigned to –CH_2–CH_2–**C**$\mathbf{H}_2$–) belonging to C_{16} with the integral of the peak related to protons at 2.69δ assigned to –CO–CH–**C**$\mathbf{H}_2$–CO–NH– belonging to PAHy. The derivatization degree was expressed as DD = (hexadecylalkyl groups/polymer repeating unit) × 100 (mol). It was found for PAHy–C_{16} a DD value of 5 mol%.

Analogously, for PAHy–PEG_{2000}–C_{16}, the DD value was 9 mol% (for C_{16} fraction) and 5 mol% (for PEG_{2000} fraction).

2.3. Micelle preparation and drug loading procedure

Hundred milligrams of PAHy–C_{16} has been mixed with 15 mg of Ketoprofen. First, the solid mixture has been damped with 0.5 ml of distilled water, then 10 ml of distilled water were added slowly thus obtaining a suspension that was, successively, sonicated for 10 min. Since the concentration of PAHy–C_{16} is greater than the value of critical aggregation concentration (CAC) (that resulted to be 23 mg/l as reported in a work in press), this suspension contains undissolved Ketoprofen and drug loaded PAHy–C_{16} micelles.

The suspension was filtered through a polycarbonate filter having a diameter of 0.45 μm in order to eliminate the undissolved amount of drug, whereas the liquid phase containing drug loaded micelles was lyophilized.

The amount of drug loaded in the micelles was determined by HPLC analysis carried out using a Varian 9012 liquid chromatograph equipped with a Rheodyne 7125 injector (fitted with a 10 μl loop), a Kontron HPLC 432 detector and a Hewlett-Packard 3394 integrator. A reverse-phase C_{18} column (μBondapak; 10 μm of 250 mm × 4.6 mm i.d., obtained from Waters) has been employed. In particular, 10 mg of drug loaded micelles were dissolved in distilled water and the solution was assayed by HPLC using a mixture CH_3CN/H_3PO_4 0.03%, pH 2.5, 45:55 v/v as eluant at 1 ml/min; the eluate was monitored at 254 nm. Drug loading value, determined in triplicate, resulted to be 10.70% w/w with a standard error within ±5%.

2.4. Liposome preparation

DMPC/DMPA (80:20, mol:mol) multilamellar vesicles (MLV) were prepared in the absence of polymers or drug at 37 °C, i.e. a temperature above the gel-to-liquid crystalline phase transition (T_m of DMPC/DMPA is 28.80 °C), starting from a chloroform/methanol (1:1 v/v) lipid stock solution. Solvents were removed under a nitrogen stream in a rotavapor and the resulting film was lyophilized. Liposomes were prepared by adding to the lipidic film 50 mM Tris buffer (pH 7.4) then heating at 37 °C and vortexing three times for 1 min. The samples were shaken for 1 h in a water bath at 50 °C to homogenize the liposomes. Afterwards, aliquots of 120 μl (7375×10^{-3} mmol of lipid) were transferred in 160 μl DSC aluminum pans and submitted to DSC analysis.

2.5. Differential scanning calorimetry

A Mettler Toledo STAR^e system equipped with a DSC-822 calorimetric cell and a Mettler TA-STAR^e software was used. The scan rate employed was 2 °C/min in the temperature range 10–45 °C. The resolution of the signal was smaller

than 0.04 μW, and the reference pan was filled with pH 7.4 Tris buffer. The calculations were performed by the Mettler STAR^e, Version 6.10 software. All samples, after the calorimetric scans, were extracted from the pan and aliquots were used to determine the amount of phospholipids by the phosphorous assay [18].

2.6. Interaction between KPF and DMPC/DMPA vesicles

To evaluate the effect of increasing amounts of KPF on lipid phase transition, lipidic films mixed with KPF were prepared. Chloroform–methanol stock solutions of phospholipids and drug were mixed to obtain various molar fractions of KPF. Organic solvents were removed under nitrogen stream in a rotavapor and the resulting film was lyophilized. Afterwards, every sample was hydrated with Tris buffer (pH 7.4) and submitted to DSC analysis. The maximum effect on the thermotropic behavior has been found for DMPC/DMPA vesicles containing a molar fraction 0.12 of KPF (sample MLV/KPF).

Beside, a kinetic experiment was also performed to investigate the transfer of KPF to liposomes. In particular, DMPC/DMPA multilamellar vesicles were incubated in a calorimetric pan, for various times (range 0–8 h) and at 37 °C, with a weighed amount of free powdered KPF.

2.7. Interaction between polymers and DMPC/DMPA vesicles

The possible interaction of all prepared samples (PAHy, PAHy–PEG_{2000}, PAHy–C_{16} and PAHy–PEG_{2000}–C_{16}), with DMPC/DMPA vesicles was investigated by DSC analysis after incubation for various times (range 0–8 h) at 37 °C.

The broadening of the peak, the appearance of a shoulder before the T_m, as well as the variation of enthalpy and temperature onset (T_{on}) have been considered as an indication of the interaction between the polymeric sample and DMPC/DMPA vesicles.

2.8. Release experiments monitored by DSC

Known amount of KPF loaded PAHy–C_{16} micelles, were added to DMPC/DMPA multilamellar vesicles in such a way as to have a molar fraction of drug respect to phospholipid equal to 0.12. The kinetics of drug release was followed at 37 °C and until 8 h of incubation. The samples were analyzed by DSC immediately after preparation by the following schedule:

(1) a first scan from 10 to 45 °C, at a heating scan of 2 °C/min, to detect the drug release;
(2) a fast cooling scan, at 4 °C/min, of the sample to 37 °C;
(3) an isothermal incubation period of 1 h at 37 °C;
(4) a fast cooling scan, at 4 °C/min, of the sample to 10 °C;
(5) a subsequent scan from 10 to 45 °C to detect the drug released after the incubation time.

This procedure was repeated several times (at least eight times) until a nearly constant drug release was observed.

3. Results and discussion

3.1. Preparation of new PAHy derivatives

PAHy derivatives containing PEG_{2000} and/or hexadecylalkyl (C_{16}) pendent groups were prepared as described in Section 2.2.2. in order to obtain new polymers having peculiar physicochemical and biological properties. Fig. 1 shows the chemical structure of starting polymer (PAHy) and its derivatives (PAHy–PEG_{2000}, PAHy–C_{16} and PAHy–PEG_{2000}–C_{16}).

In effect, PEG residues can increase, potentially, the biocompatibility and reduce the uptake by the reticuloendothelial system of the resulting polymeric derivatives, thus prolonging their circulation time. On the other hand, the presence of hexadecylalkyl groups gives, to PAHy–C_{16} and PAHy–PEG_{2000}–C_{16} samples, the opportunity to arrange themselves as supramolecular structures, i.e. polymeric micelles. We suppose that, in aqueous medium, PAHy and PEG chains (when present) could be exposed to the external phase, whereas C_{16} residues could form an inner hydrophobic core. Due to its peculiar structure in aqueous medium, PAHy–C_{16} sample resulted able to entrap water insoluble molecules such as Ketoprofen chosen as a model drug.

In order to evaluate the ability of all prepared samples (PAHy, PAHy–PEG_{2000}, PAHy–C_{16} and PAHy–PEG_{2000}–C_{16}) to interact with a biomembrane model, formed by DMPC/DMPA multilamellar vesicles, a systematic calorimetric study has been performed and discussed afterwards. The same approach has been employed to study the uptake by DMPC/DMPA liposomes of Ketoprofen released from PAHy–C_{16} micelles.

3.2. Polymers–DMPC/DMPA vesicles interaction

In Figs. 2–5 the calorimetric profiles obtained by incubating at 37 °C all prepared samples (PAHy, PAHy–PEG_{2000}, PAHy–PEG_{2000}–C_{16} and PAHy–C_{16}) with DMPC/DMPA multilamellar vesicles (MLV) for various times, are reported. To compare the effect exerted by the investigated polymers on MLV, the values of $\Delta(\Delta H)$% or $\Delta(\Delta T_{on})$% have been reported as a function of incubation time (see Figs. 6 and 7, respectively). $\Delta(\Delta H)$% is the percentage decrease of the enthalpy (during the gel-to-liquid crystalline phase transition) due to the interaction between the investigated polymers and DMPC/DMPA vesicles. $\Delta(\Delta T_{on})$% is the percentage decrease of the ΔT_{on} that is the difference between the T_{on} (i.e. temperature onset) of DMPC/DMPA liposomes incubated with the polymers and the T_{on} of liposomes alone expressed in degree Celsius. T_{on} is defined as the intersection point of the baseline before transition and the inflectional tangent.

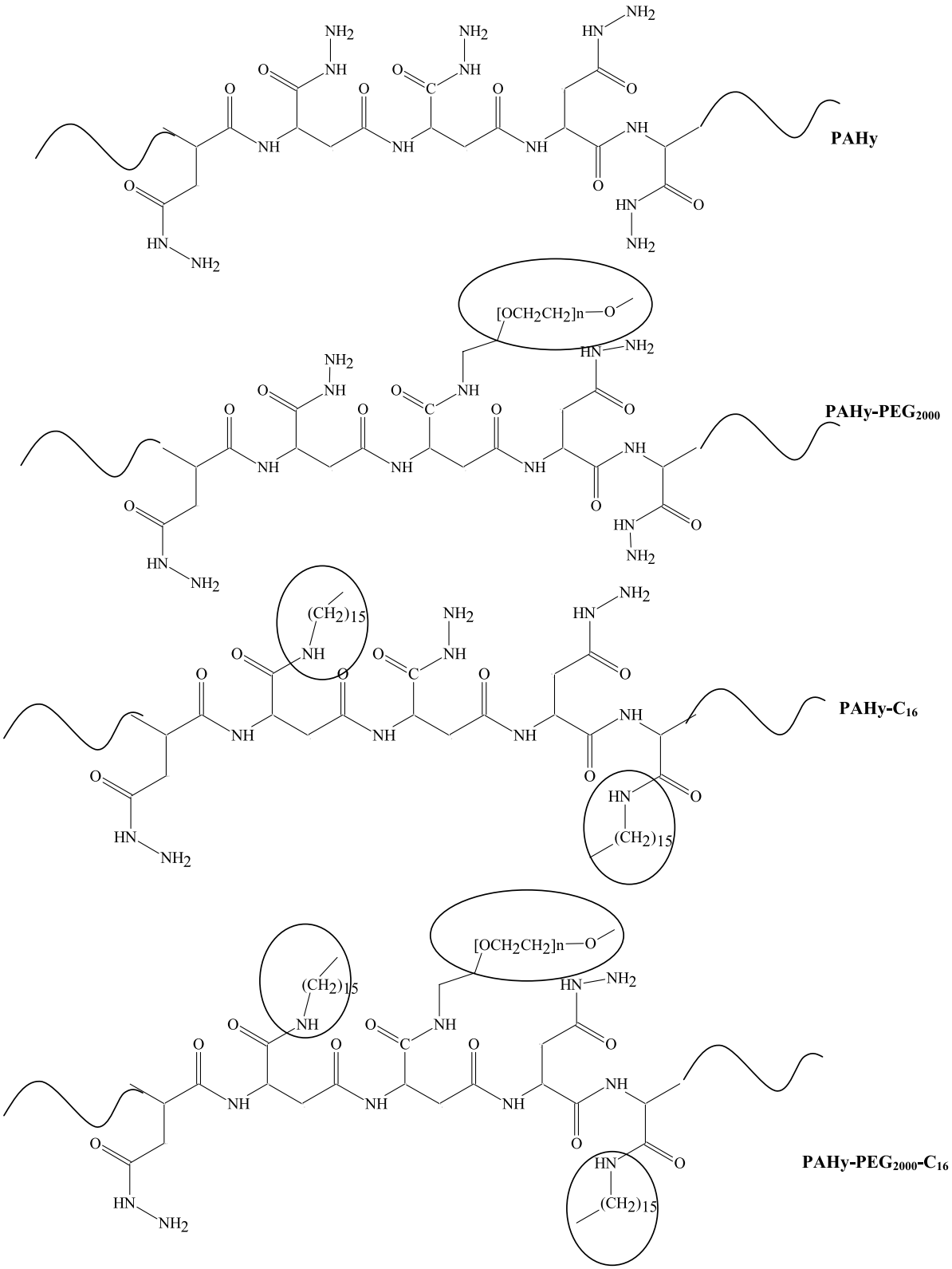

Fig. 1. Chemical structure of PAHy, PAHy–PEG_{2000}, PAHy–C_{16} and PAHy–PEG_{2000}–C_{16}.

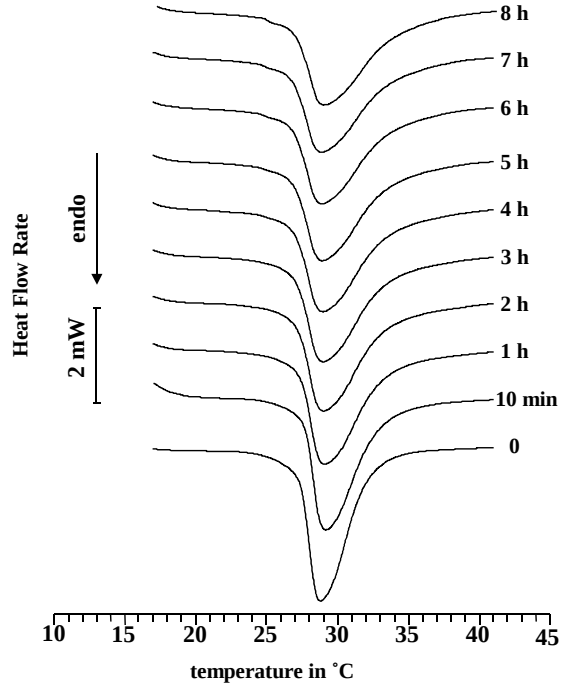

Fig. 2. Calorimetric curves of DMPC/DMPA multilamellar vesicles incubated at 37 °C with PAHy for various incubation times.

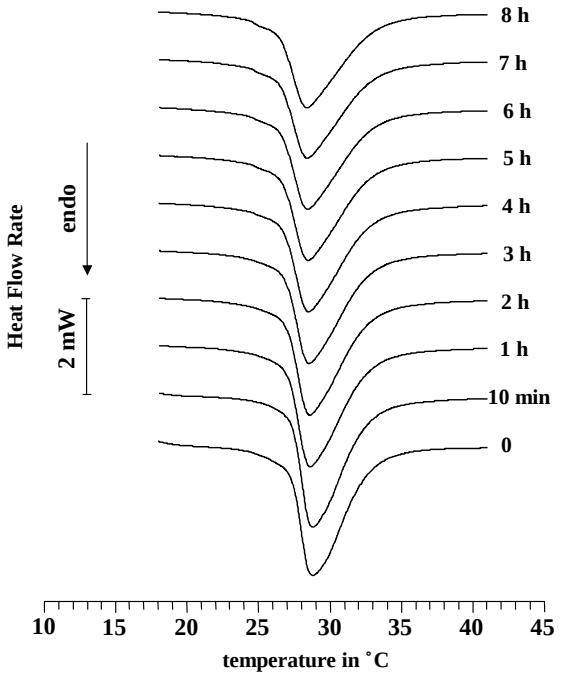

Fig. 4. Calorimetric curves of DMPC/DMPA multilamellar vesicles incubated at 37 °C with PAHy–PEG_{2000}–C_{16} for various incubation times.

It is evident (Fig. 2) as PAHy is able to broaden the calorimetric peak with a contemporary shift of the T_{on} towards lower values (see Fig. 7), whereas the enthalpy changes remain nearly constant as a function of incubation time (see Fig. 6).

The introduction of a hydrophilic group (PEG_{2000}) in the polymeric structure, causes a less effect on the shape of the calorimetric curves (Fig. 3) meaning that an increase in polymer solubility decreases its ability to permeate the multilamellar lipidic structure; as a consequence no significant

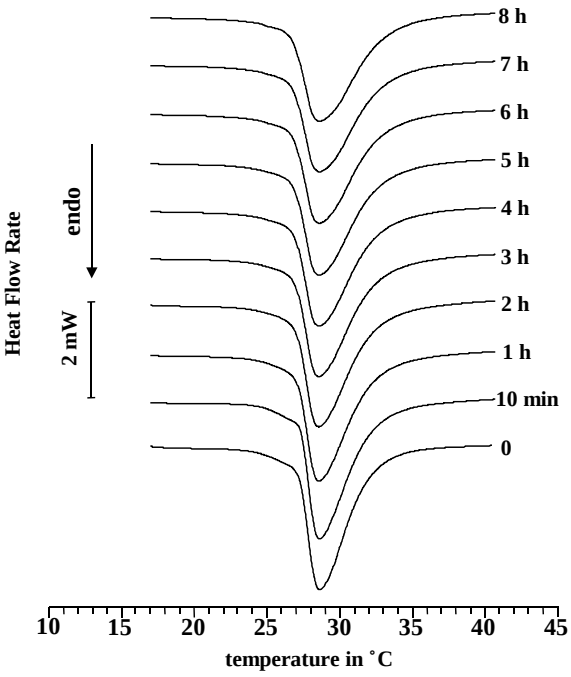

Fig. 3. Calorimetric curves of DMPC/DMPA multilamellar vesicles incubated at 37 °C with PAHy–PEG_{2000} for various incubation times.

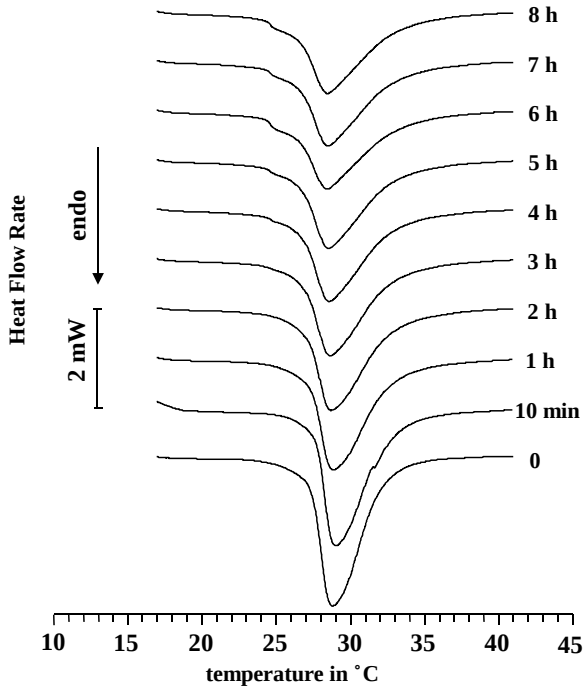

Fig. 5. Calorimetric curves of DMPC/DMPA multilamellar vesicles incubated at 37 °C with PAHy–C_{16} for various incubation times.

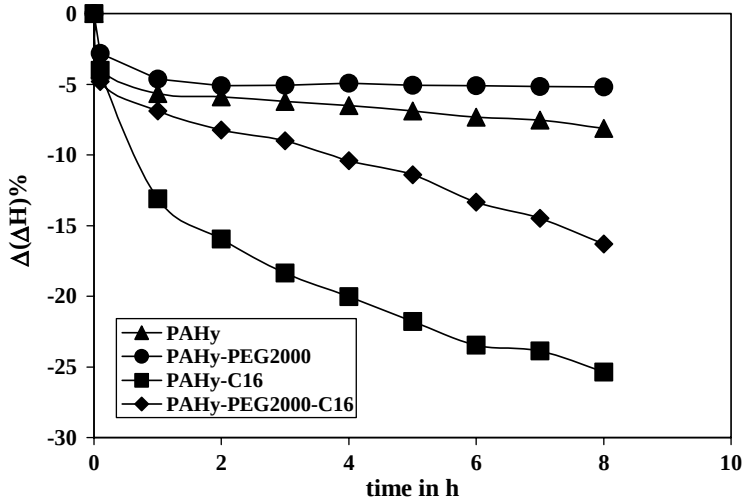

Fig. 6. Enthalpy changes observed for aqueous dispersion of DMPC/DMPA multilamellar vesicles incubated at 37 °C with all investigated samples as a function of incubation time.

variation on ΔH and ΔT_{on} has been observed as a function of incubation time (see Figs. 6 and 7).

The presence of C_{16} residues in the polymeric backbone together with PEG_{2000} chains (sample PAHy–PEG_{2000}–C_{16}), causes an increase in the fluidifying effect on the multilamellar vesicles in fact, as reported in Figs. 6 and 7, the values of $\Delta(\Delta H)$% or $\Delta(\Delta T_{on})$%, respectively, undergo a more pronounced reduction as a function of incubation time. This result suggests that the introduction of hydrophobic residues causes a destabilizing effect on the lipidic membrane due to a potential penetration of C_{16} chains. Obviously, the presence of C_{16} residues alone (sample PAHy–C_{16}), causes the greatest variation in the values of enthalpy and temperature onset (see Figs. 6 and 7). On the other hand, after 6 h of incubation with PAHy–C_{16} sample, a shoulder before the T_m appears also (Fig. 5) thus confirming that the interaction of PAHy–C_{16} with DMPC/DMPA vesicles is greater than that of others investigated polymers.

3.3. KPF–DMPC/DMPA vesicles interaction and KPF release from PAHy–C_{16} micelles

The interaction of KPF with DMPC/DMPA multilamellar vesicles was studied by preparing liposomes loaded with various molar fractions of drug so to determine the maximum of the interaction between drug and MLV. The effect caused by drug on MLV was proportional to the amount of KPF loaded into DMPC/DMPA vesicles (data not reported). Fig. 8

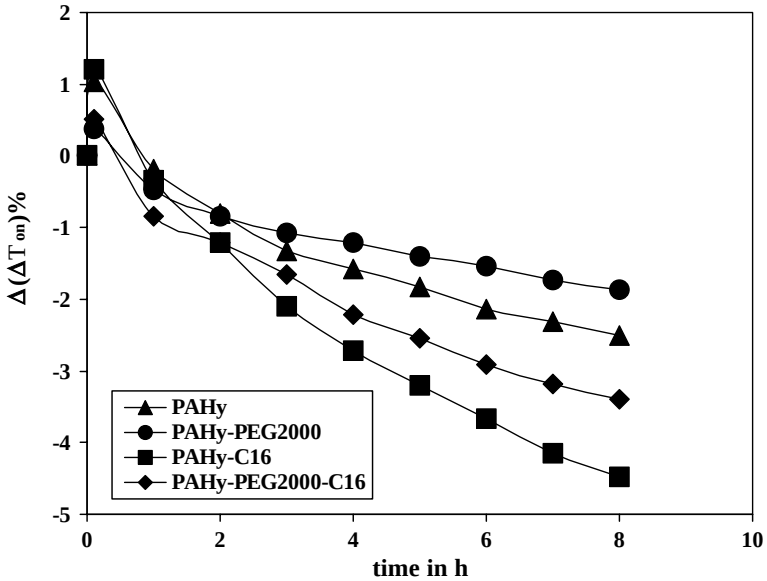

Fig. 7. Temperature shift observed for aqueous dispersion of DMPC/DMPA multilamellar vesicles incubated at 37 °C with all investigated samples as a function of incubation time.

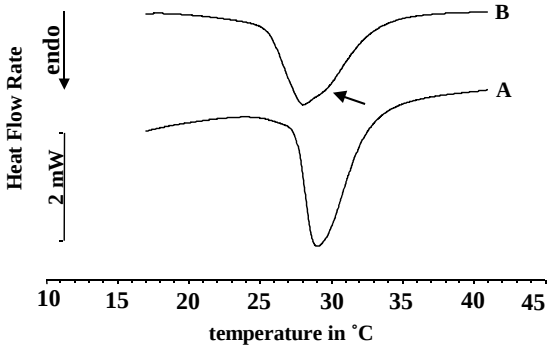

Fig. 8. Comparison between (A) calorimetric curve of pure multilamellar vesicles of DMPC/DMPA and (B) multilamellar vesicles of DMPC/DMPA containing KPF (sample MLV/KPF; molar fraction 0.12 with respect to phospholipids) prepared as described in Section 2.6.

reports the calorimetric curve of pure DMPC/DMPA vesicles compared with that of DMPC/DMPA vesicles containing a 0.12 molar fraction of KPF (sample MLV/KPF). It was chosen this molar fraction because it gave the highest effect respect to the others; it is evident, in the presence of KPF, the occurrence of a contemporary broadening of the peak, a shift of the T_m to a lower value and a phase separation (evidenced by the appearance of a shoulder after the T_m).

In previous papers, the interaction between non steroidal anti-inflammatory drugs and liposomes was studied and only a "fluidifying" effect, caused by the intercalation of drug molecules in the ordered lipid structure was observed [22–26]. This effect was manifested by the decrease of the transitional temperature of the lipid bilayer without showing any phase separation by employing only neutral lipids of dimyristoylphosphatidylcholine or dipalmitoylphosphatidylcholine. Therefore, the phase separation, observed when negatively charged vesicles are employed (DMPC/DMPA), could be due to the interaction of KPF with the charged fraction (20% of PA) of the multilamellar vesicles.

Fig. 9 reports the calorimetric curves obtained by incubating at 37 °C for various times a fixed amount of free powdered KPF (0.12 molar fraction respect to phospholipids) with DMPC/DMPA multilamellar vesicles. In this way, it is possible to study the migration of drug to the lipidic bilayer that is in a disordered state, being the experimental temperature greater than T_m. It is evident, by comparing the calorimetric curves obtained for different incubation times (range 0–8 h) with the curve obtained for the maximum interaction (dotted curve; sample MLV/KPF) as the KPF is able to penetrate the membranes causing the T_{on} shift and the appearance of a shoulder after the T_m value, reinforcing our opinion that this shoulder can be employed as an indication of the interaction between drug and lipids.

The transfer of free KPF to DMPC/DMPA vesicles was compared with the uptake by DMPC/DMPA liposomes of drug released from PAHy–C_{16} sample. In fact, considering that PAHy–C_{16} arranges oneself as micellar structure and it

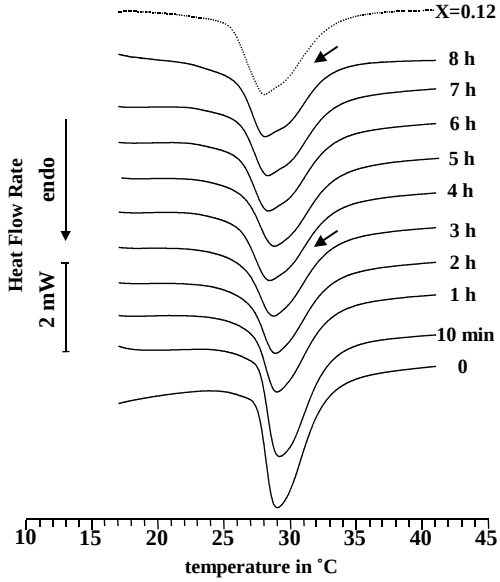

Fig. 9. Calorimetric curves obtained by incubating at 37 °C for various times, free powdered KPF with DMPC/DMPA multilamellar vesicles. An amount of KPF was used to obtain a molar fraction of 0.12 with respect to phospholipids.

is able to interact with liposomes more than others prepared polymers, we have chosen this sample to entrap Ketoprofen and its ability to release this drug to the biomembrane model has been evaluated.

Fig. 10 reports the calorimetric curves obtained by incubating at 37 °C for various times drug loaded PAHy–C_{16} micelles with DMPC/DMPA vesicles. In the experiment it was

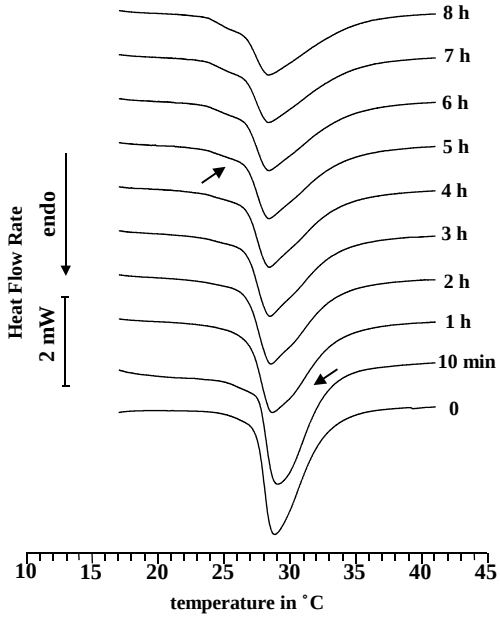

Fig. 10. Calorimetric curves obtained by incubating at 37 °C for various times, KPF containing PAHy–C_{16} micelles with DMPC/DMPA multilamellar vesicles. An amount of micelles was used to obtain a molar fraction of 0.12 of drug with respect to phospholipids.

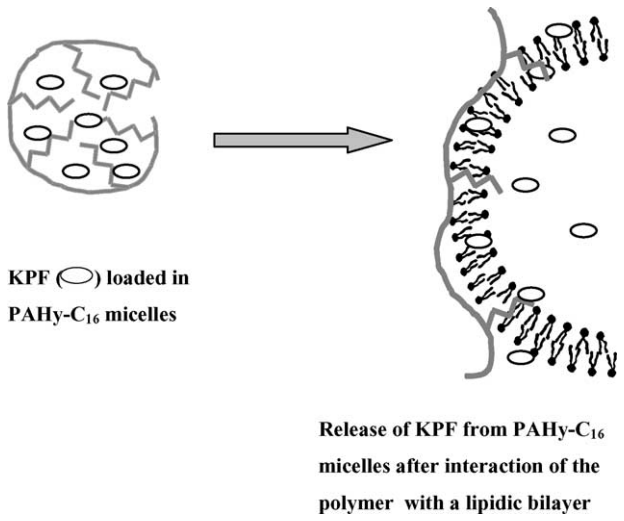

Fig. 11. Schematic representation of the probable mechanism of KPF release from PAHy–C_{16} micelles to DMPC/DMPA multilamellar vesicles.

employed an amount of drug loaded micelles to obtain a molar fraction 0.12 of drug respect to phospholipids. It appears evident as, after one hour from the beginning of the experiment, the characteristic shoulder ascribed to the interaction of KPF with lipidic vesicles appears, but in time this shoulder becomes smoother. We can suppose that, at the beginning of the release process, the delivered KPF penetrates the lipidic membrane in which initially it is inhomogeneously distributed and the shoulder appears more evident. In time, the distribution of drug in the lipidic membrane is more homogeneous and the shoulder is less evident. Besides, after 5 h of incubation, a small shoulder appears before the T_m imputable to the polymer–vesicles interaction. A broadening of the peak and a decrease of the T_{on} are also detectable, thus confirming the interaction between the polymer and the lipidic bilayer.

The obtained data suggest that the probable mechanism of the drug release from PAHy–C_{16} micelles could involve a penetration of C_{16} chains into the lipidic bilayer with a following delivery of drug molecules which, becoming free, are able to penetrate the membrane (see Fig. 11).

Therefore, PAHy–C_{16} micelles besides their ability to transport lipophilic drugs, are also able to promote their release and to favor, potentially, their penetration across the biological membranes.

4. Conclusions

α,β-Polyaspartylhydrazide (PAHy) and some of its derivatives (PAHy–PEG_{2000}, PAHy–C_{16} and PAHy–PEG_{2000}–C_{16}) are able to interact with a biomembrane model, formed by liposomes of DMPC/DMPA, but the extent of this interaction depends on the balance between hydrophilic and hydrophobic moieties. The maximum interaction has been observed for PAHy–C_{16} sample that, on the other hand, resulted in the formation of micelles in aqueous medium and entrapment of lipophilic drugs such as Ketoprofen.

DSC analysis showed that PAHy–C_{16} micelles are able to promote drug uptake by the biomembrane model. We think that the polymeric micelles migrate through the aqueous medium, they interact with the lipidic vesicles and they release the drug “in situ”. Then, the entrapment of Ketoprofen in PAHy–C_{16} micelles could improve the localized release close to the biological target.

It has been demonstrated that DSC analysis allows to study the drug release and its uptake by a biomembrane model. The employed experimental approach can be considered as “a nearly in vivo” test more profitable than the classical dissolution “in vitro” test because it allows to evaluate the drug uptake by a model that simulates the cell membrane.

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