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New amphiphilic derivatives of poly(ethylene glycol) (PEG) as surface modifiers of colloidal drug carriers. III. Lipoamino acid conjugates with carboxy- and amino-PEG$_{5000}$ polymers

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ABSTRACT

Within a research directed to developing new polymeric materials, suitable for decorating the surface of colloidal drug carriers, PEG$_{5000}$ polymers containing a free carboxyl or amine group at one end were conjugated to an α-lipoamino moiety (LAA). The conjugates were characterized by FT-IR, ^{1}H-NMR, and MALDI-TOF mass spectrometry. They showed the same profile of solubility as the parent PEGs in water and in some polar and apolar solvents of pharmaceutical use. Representative terms showed to be well tolerated when incubated with Caco-2 or L929 cell cultures.

Dedicated differential scanning calorimetry (DSC) studies were performed to prove the interaction of increasing molar fractions of the PEG$_{5000}$–LAA conjugates with dipalmitoylphosphatidylcholine (DPPC) bilayers, to gain information about their possible incorporation in drug nanocarriers. While the parent PEGs affected only the superficial structure of bilayers, the amphiphilic PEG–LAA conjugates induced a perturbing effect on the thermotropic behavior of DPPC liposomes, according to the structure of the linked LAA residue. A molar concentration of these PEG–LAA between 5 and 10% was individuated as the most suitable to produce stable vesicles.

1. Introduction

In a recent research project, new amphiphilic derivatives of poly(-ethylene glycol) (PEG), created by conjugating carboxy or amine PEG derivatives to one or two lipoamino acid (LAA) residues have been synthetized [1]. The aim of the whole study is to produce amphiphilic forms of the hydrophilic PEG polymer that should be able to anchor to and cover the surface of colloidal (nano) drug carriers. Such conjugates can represent an alternative to phospholipid-PEGs in the preparation of long-circulating (stealth) liposomes and especially of stealth lipid-based (SLN or NLC) and polymeric nanocarriers (nanoparticles, micelles, nanocapsules, etc.).

It is well known that the in vivo efficacy of colloidal drug carriers is usually limited by their rapid removal from the systemic circulation. This protective mechanism first involves tagging of nanoparticle surface by some plasmatic proteins called opsonins; the consequent activation of complement drives the recognition of the *non-self-entities* by circulating macrophages or located in specific organs (liver, spleen, lungs, etc.) – the so-called 'mononuclear phagocyte system' (MPS) – which will ultimately degrade extraneous materials [2,3].

Surface decoration of colloidal carriers with hydrophilic macromolecules, that prevent the opsonization and hinder the carriers to MPS cells, has arisen to a gold standard method to ensure a circulation time in the bloodstream long enough to allow the controlled or targeted release of the carried actives [4–8]. In particular, PEGylation has attracted a wide attention and investigations; although the potential limits of PEG, due for instance to immunological or toxicological issues [3,9,10], the few examples of clinically employed stealth systems make advantage of a limited number of well characterized phospholipid PEG derivatives [5,6,11,12].

Several authors have investigated the strategy of derivatizing PEG polymers with different phospholipids or other lipids, such as diglycerides, cholesterol, ceramides and phosphatidic acid. All these lipophilic modifiers are particularly valid to anchor the PEG residue to phospholipid-based vesicles, such as liposomes. Conversely, they can encounter some steric problems with polymer-based nanocarriers: the lipid portion of the above PEG derivatives has in fact a considerable size, that may scarcely fit a lipidic or polymeric matrix, ultimately reducing the stability of the whole system and the permanence of PEG covering once injected in the bloodstream. The last thoughts prompted us to

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discover alternative amphiphilic modifiers for colloidal drug carriers and, in particular, to investigate the potentiality of various polymer conjugates in which: i) LAAs were linked to either amino- or carboxy-PEGs; ii) the type and number of LAA moieties were changed; and, iii) the molecular weight of the parent PEG polymers varied between 2000 and 5000.

This last parameter has received a certain attention in the recent literature. It could be intuitive that the higher the molecular weight of PEG, the longer its hydrophilic portion, and the greater its 'covering' effect on the (nano)particle surface and the efficacy of prolonging the permanence of the colloidal carries in the bloodstream [13]. Although it has been shown to be difficult to delineate a linear correlation between the molecular weight of PEG and its efficacy in producing 'sterically stable' nanocarriers, a general conclusion is that a molecular weight between 1000 and 5000 Da is the more suitable for producing good and stable stealth systems [13,14].

In recent papers we have described different series of mono- and bis(carboxy)- and (amino)PEG_{2000} conjugates with LAAs [15,16]. Some of these conjugates have been successfully validated as surface modifiers of colloidal drug carriers, like liposomes and lipid nanoparticles [17–19].

LAAs (Fig. 1) are α-amino acids bearing an alkyl side chain of different length in 2-position. They represent a class of compounds which merge the physico-chemical features of lipids and amino acids: they in fact possess a marked lipophilicity due to the hydrophobic tail, while keeping the polar characteristics of amino acids [20,21].

Such bi-functionality allows to link the LAAs to drugs or other compounds having a wide variety of reactive groups. The resulting conjugates would possess an amphiphilic character that may assist their interaction with cell membranes and crossing through biological barriers [22,23]. In particular, drug–LAA conjugates have been shown to ensure a complex and multi-level interaction with an anisotropic 3-D biomembrane model, represented of 1,2-dipalmitoylphosphatidylcholine (DPPC) multilamellar liposomes (MLV) [24].

On these bases, in the present paper the synthesis and characterization are reported of PEG–LAA conjugates obtained from monofunctionalized carboxy- and amino-PEG polymers with a molecular weight of 5000 Da. To assess the influence of an increasing lipophilicity on the physico-chemical properties of the PEG conjugates, LAA bearing an alkyl side chain between 16 and 20 carbon atoms (m = 13–17, Fig. 1) was tested.

The structure of the synthesized conjugates was confirmed by ^{1}H-NMR analysis, FTIR spectroscopy and MALDI-TOF mass spectrometry. Specific studies were performed by differential scanning calorimetry (DSC) to evaluate the interaction mode of PEG_{5000}–LAA conjugates with DPPC MLV, and to gain some preliminary information about their potential applications as surface coating of colloidal drug carriers. Finally, an in vitro cytotoxicity assay was performed to confirm the tolerability of these conjugates on mammalian cells.

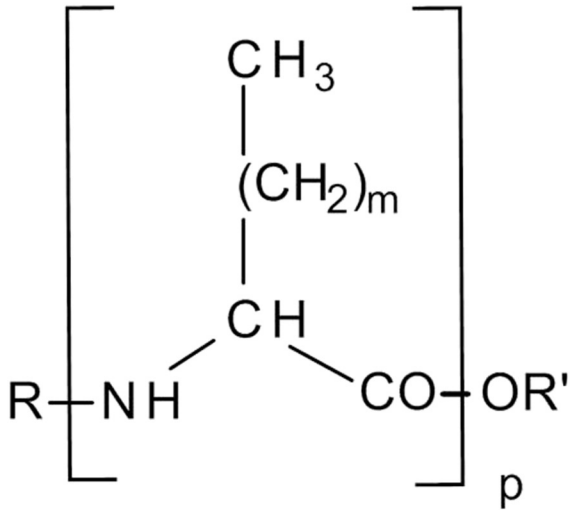


Fig. 1. General structure of LAAs. m = 1–17; p ≥ 1; R = H; Boc; Fmoc; other protecting groups; R' = OH; OCH_3.

2. Experimental

2.1. Materials

The α-methoxy-ω-carboxy-PEG_{5000} (**1**) and methoxy-PEG-40 monostearate (**3**) were purchased from Sigma-Aldrich Chimica srl (Milan, Italy); α-methoxy-ω-amino-PEG_{5000} (product code PEG1154) was purchased from Iris Biotech GmbH (Marktredwitz, Germany). DPPC was a product from Genzyme Pharmaceuticals (Liestal, Switzerland). Eagle's Minimum Essential Medium (MEM) and Dulbecco's Modified Eagles Medium (DMEM) were products from Gibco BRL (Life Technologies, Milan, Italy); pH 7.4 phosphate buffered saline (PBS) and 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chimica srl (Milan, Italy); all the other reagents and solvents were commercially available; solvents were dried before use by distillation according to standard methods [25] and stored over 4 Å molecular sieves activated at 400 °C for at least 24 h.

^{1}H-NMR spectra were recorded on a Varian Inova instrument operating at 500 MHz using $CDCl_3$ as solvent; the chemical shifts are given in ppm from TMS as the internal standard. IR spectra were acquired using an FTIR Jasco 1700 instrument; test samples were dissolved in chloroform and spread on KBr disks. Sixty-four scans were signal-averaged with a resolution of 2 cm^{-1}, at room temperature. MALDI-TOF mass spectrometry was performed using a Voyager STR instrument or with a 4800 mass spectrometer (Applied Biosystems, Framingham, MA, USA). The MALDI matrix was 0.2 M 2,5-dihydroxybenzoic acid (DHB) in water/MeOH, 1:4 (v/v). The samples were dissolved in the same solvent mixture at a concentration of 5 mg/ml, mixed with the matrix and dried in air. The spectra were acquired in both linear and reflected modes. Melting points of the starting polymers and conjugates were measured using a Mettler DSC12E calorimeter, using an empty aluminum pan as the reference (see Table 1).

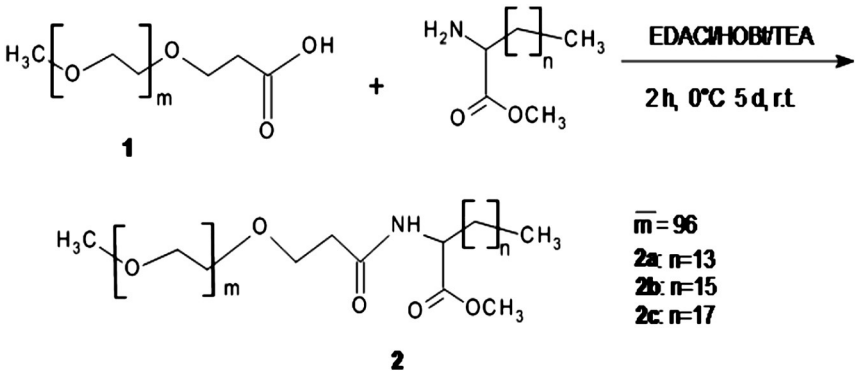
2.2. Synthetic procedures

2.2.1. Amide conjugates of α-methoxy-ω-carboxy-PEG_{5000} with LAAs (**2a–c**)

1-Hydroxybenzotriazole (HOBt), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDAC) and triethylamine (TEA) were added to a solution of **1** in dry dichloromethane (DCM); the mixture was kept at 0 °C for 2 h, thereafter equimolar amounts of the appropriate LAA methyl ester hydrochloride [26] and TEA were added. The mixture was left under magnetic stirring at room temperature for 5 days (Scheme 1). The reaction course was checked out by silica gel TLC, using a DCM/MeOH, 8:2 (v/v) eluent mixture; spots were visualized by treatment with a ninhydrin ethanol solution. At the end, the solvent was removed off under reduced pressure and the residue **2a–c** was

Table 1
Melting point of the starting PEGs and of the synthesized conjugates.

Conjugate or starting polymer	Melting point (°C)
Carboxy-$mPEG_{5000}$ **1**	58.4
$mPEG_{5000}$C–LAA16 **2a**	57.5
$mPEG_{5000}$C–LAA18 **2b**	57.4
$mPEG_{5000}$C–LAA20 **2c**	58.0
Amino-$mPEG_{5000}$ **3**	56.9
$mPEG_{5000}$N–LAA16 **4a**	54.0
$mPEG_{5000}$N–LAA18 **4b**	51.7
$mPEG_{5000}$N–LAA20 **4c**	50.3



Scheme 1. Amide conjugates of α-methoxy-ω-carboxy-PEG_{5000} with LAAs (**2a–c**).

purified by semipreparative TLC on silica gel plates, using the same above eluent mixture.

*2.2.2. Amide conjugates of α-methoxy-ω-amino-PEG_{5000} with LAAs (**4a–c**)*

Dicyclohexylcarbodiimide (DCC; 1 M solution in DCM) and 4-dimethylaminopyridine (DMAP) were added to an equimolar solution of **3** and the chosen N-Boc-LAA [26] in dry DCM (5 ml). The mixture was kept under stirring at room temperature for 3 days (Scheme 2) and the reaction course was checked out by silica gel TLC, using a DCM/methanol, 85:15 (v/v) eluent mixture; spots were visualized by treatment with a ninhydrin ethanol solution. The mixture was filtered to remove the produced dicyclohexylurea (DCU), treated repeatedly with cold diethyl ether, and the solvent evaporated under reduced pressure to stop the reaction. The residue was finally purified through semipreparative TLC on silica gel plates, using a DCM/MeOH eluent mixture (85:15, v/v). The Boc protecting group was removed by stirring with a solution of trifluoroacetic acid (TFA) in DCM (1:1, v/v), at room temperature for 2 h. The solvent was evaporated under vacuum, after several washes with DCM to remove all TFA used. The final products **4a–c** were finally identified by TLC using the same above eluent mixture.

*2.3. Characterization of conjugates **2** and **4***

2a: methyl-2-{3-[α-methylpoly(oxyethylene)]-propanoyl}amino-hexadecanoate: IR (KBr, cm^{-1}): 2884, 1737, 1669, 1648, 1466, 1347, 1281, 1244, 1152, 1113; ^{1}H-NMR ($CDCl_3$, ppm, δ): 4.50 (br, α-CH), 3.72 (s, $COOCH_3$), 3.64 (m, OCH_2CH_2O), 3.44 (m, OCH_2CH_2), 3.37 (s, OCH_3), 2.53 (m, $COCH_2CH_2CO$), 1.65 (m, CH_2), 1.24 (m, LAA CH_2), 0.87 (t, ω-CH_3).

2b: methyl-2-{3-[α-methylpoly(oxyethylene)]-propanoyl}amino-octadecanoate: IR (KBr; cm^{-1}): 2883, 1739, 1684, 1654, 1548, 1488, 1363, 1298, 1262, 1155, 1112, 1071; ^{1}H-NMR ($CDCl_3$, ppm, δ): 4.53 (br, α-CH), 3.72 (s, $COOCH_3$), 3.62 (m, OCH_2CH_2O), 3.45 (m, OCH_2CH_2), 3.35 (s, OCH_3), 2.53 (m, $COCH_2CH_2CO$), 1.70 (m, CH_2), 1.22 (m, LAA CH_2), 0.86 (t, ω-CH_3).

2c: methyl-2-{3-[α-methylpoly(oxyethylene)]-propanoyl}amino-eicosanoate): IR (KBr, cm^{-1}): 2952, 2881, 2798, 1742, 1684, 1649, 1546, 1486; 1363, 1297, 1250, 1160, 1110, 1006; ^{1}H-NMR ($CDCl_3$, ppm, δ): 4.50 (br, α-CH), 3.72 (s, $COOCH_3$), 3.63 (m, OCH_2CH_2O), 3.47–3.43 (m, OCH_2CH_2), 3.36 (s, OCH_3), 2.53 (m, $COCH_2CH_2CO$), 1.66 (m, CH_2), 1.23 (m, LAA CH_2), 0.86 (t, ω-CH_3).

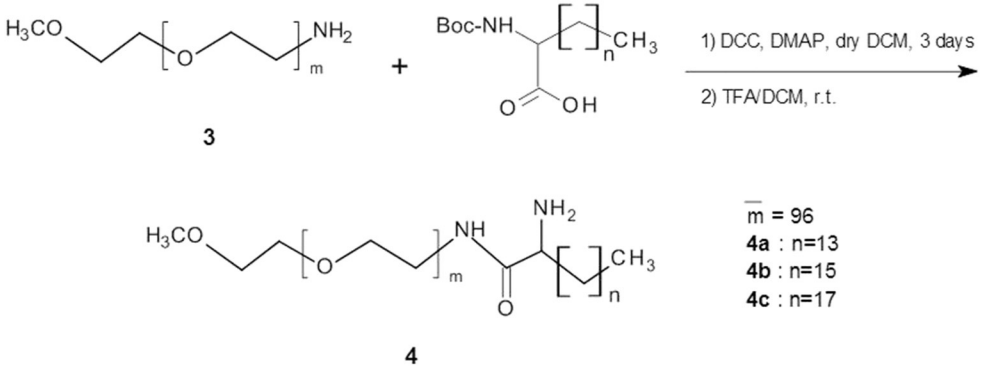
4a: 2-amino-hexadecanamide,N-[α-methylpoly(oxyethylene): IR (KBr; cm^{-1}): 2922, 2870, 1673, 1452, 1347, 1277, 1240, 1144, 1101; ^{1}H-NMR ($CDCl_3$, ppm, δ): 4.10 (m, α-CH), 3.55 (m, OCH_2CH_2O), 3.42 (m, $OCH_2\underline{CH_2}NH$), 3.34 (s, OCH_3), 1.59 (m, CH_2), 1.29 (s, PEG–CH_2), 0.87 (t, ω-CH_3).

4b: 2-amino-octadecanamide,N-[α-methylpoly(oxyethylene): IR (KBr; cm^{-1}): 2930, 2875, 2866, 1673, 1454, 1347, 1280, 1240, 1101, 1059; ^{1}H-NMR ($CDCl_3$, ppm, δ): 4.13 (m, α-CH), 3.59–3.54 (m, OCH_2CH_2O), 3.46–3.41 (m, $OCH_2\underline{CH_2}NH$), 3.35 (s, OCH_3), 1.61–1.54 (m, CH_2), 1.27 (s, LAA CH_2), 0.87 (t, ω-CH_3).

4c: 2-amino-eicosadecanamide,N-[α-methylpoly(oxyethylene): IR (KBr; cm^{-1}): 2923, 2872, 1672, 1467, 1453, 1347, 1280, 1244, 1146, 1098, 1057; ^{1}H-NMR ($CDCl_3$, ppm, δ): 4.07 (m, α-CH), 3.57 (m, OCH_2CH_2O), 3.44 (m, $OCH_2\underline{CH_2}NH$), 3.35 (s, OCH_3), 1.59–1.55 (m, CH_2), 1.23 (s, LAA–CH_2), 0.87 (t, ω-CH_3).

2.4. Solubility determination of PEG–LAA conjugates

The solubility profile of the synthesized conjugates was assayed at room temperature in several solvents of pharmaceutical and industrial interests: distilled water, isotonic phosphate buffered saline (PBS) at pH 7.4, ethanol, acetone, dimethylsulfoxide, ethyl acetate, and dichloromethane. Increasing amounts of each conjugate were added to 5 ml of solvent and slowly stirred for 30 min. The limit of solubility of each sample was assessed by turbidimetry, performed at 550 nm with a Shimadzu UV-1601 instrument.



Scheme 2. Amide conjugates of α-methoxy-ω-amino-PEG_{5000} with LAAs (**4a–c**).

2.5. DSC studies

2.5.1. Liposome preparation

The polymer conjugate to be tested was added at different molar concentrations (3, 5 or 10%) to pure DPPC (5 mg). Both ingredients were dissolved in a round-bottomed glass tube with 1 ml of a 1:1 chloroform/methanol mixture and the solvents were removed under a nitrogen flow while gently rotating the tube. The obtained thin film was kept for 8 h in an oven under high vacuum at 30 °C (Büchi Labortechnik AG; Flawil, Switzerland) and then stored at −20 °C until use. MLVs were produced by hydrating the dry film with 300 μl of a 0.13 N phosphate buffer solution (pH of 7.4) [27]. The suspension was alternately warmed in a water bath at about 55 °C and vortex-mixed for 2 min; the whole cycle was repeated for three times. The vesicle suspensions were then kept for 1 h at 60 °C to reach a thermal equilibrium among the phospholipid bilayers.

2.5.2. DSC analysis

Experiments were performed using a Mettler DSC12E calorimeter connected to a Haake D8-G thermocryostat (Haake Messtechnik, Karlsruhe, Germany). A sample of pure indium was used to calibrate the instrument. The DSC scans had an accuracy of ±0.4 °C, and a reproducibility and resolution of 0.1 °C. Forty microliters of each liposome sample was sealed in an aluminum pan and subjected to 3 cycles of analysis (in heating and cooling modes), at a heating/cooling rate of 2 °C/min in a 15–60 °C temperature range. As a reference, a pan was filled with 40 μl of the same buffer solution used for liposome production. The experimental data were compared for the different conjugate molar concentrations using, as reference thermotropic parameters the changes of phase transition temperature (ΔT%) and the variation of the enthalpy changes associated to this transition [Δ(ΔH)], compared to pure DPPC vesicles [28]. The value of ΔT% was defined as: [Tm − T°m] × 100, where T°m and Tm are the transition temperatures respectively of pure DPPC vesicles and of liposomes loaded with the various molar concentrations of the starting PEGs or PEG–LAA conjugates. A similar equation was used to calculate the values of [Δ(ΔH)%], by comparing the values of ΔH for empty liposomes with those produced with the tested commercial polymers and conjugates.

2.6. Cytocompatibility test

2.6.1. Cell cultures

Human epithelial colorectal adenocarcinoma cells (Caco-2), obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured in MEM supplemented with 10% fetal calf serum (FBS, Gibco, USA), 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, streptomycin (50 μg/ml), and penicillin (50 U/ml). They were incubated at 37 °C in an atmosphere of 5% CO_2 in air. Cells from confluent cultures were detached by using trypsin (Sigma) (0.25%)–EDTA (1 mmol/l) and seeded to determine the biocompatibility of the tested compounds.

L929 mouse fibroblasts (ATCC, Rockville, MD, USA) were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in an atmosphere of 5% CO_2 in air. During the logarithmic growth phase, cells were trypsinized using 0.25% trypsin and resuspended in fresh medium with FBS and antibiotics.

2.6.2. MTT assay

Caco-2 and L929 cells were seeded at 1.5×10^4 cells per well in a 96-well flat-bottomed microplate, as previously reported [29]. Cells were incubated at 37 °C in a humidified 5% CO_2–95% air mixture and treated with different concentrations (1, 10, and 100 μM) of freshly prepared **2b** and **4b** solutions in the relevant culture medium (MEM or DMEM). After 48 h, 20 μl of MTT (0.5% in PBS) was added to each microwell for 4 h. The supernatant was then removed and replaced with 100 μl of DMSO. The optical density of each well sample was measured with a microplate spectrophotometer reader (Titertek Multiskan, DAS, Italy) at λ = 550 nm. The results were reported as percentage of cell viability with respect to untreated cells (control), whose absorbance was considered to be due to 100% viable cells. Each sample was tested in quadruplicate.

3. Results and discussion

3.1. Conjugate characterization

Our wide research program is aimed at discovering alternative surface modifiers for colloidal drug carriers than phospholipid-PEG derivatives. Different anchoring moieties than phospholipids, in facts, can become advantageous for grafting with PEG residues the surface of nanocarriers having a lipid or polymeric nature, like nanoparticles, nanocapsules, micelles, SLN, and NLC. In particular, in those systems showing a more packed structure than the phospholipid bilayers of liposomes, such as polymer-based nanoparticles, the phospholipid moiety of DSPE–PEG or analogous derivatives can embody a steric hindrance to a stable attachment of the hydrophilic polymer to the particle surface.

Our attention as possible lipid modifiers of PEGs was oriented towards LAAs. These promoieties have been tested in the past to modify the properties of many biologically active compounds [30–32]. Interestingly, due to their amphiphilic nature, LAA–drug conjugates have shown to be able to develop a more complex interaction with phospholipid bilayers than simple alkyl drug derivatives [24], thus becoming a potential mean to ensure a profound and relatively stable fixation of the PEG residues to liposome or nanoparticle structures.

Different structural variables were planned in the general project, as well as in the synthesis described in the present paper. As discussed before, starting PEGs with a molecular weight of 5000 Da were selected in this paper, and compared to the corresponding conjugates of LAA with PEG_{2000} polymers [15,16]. PEGs with this order of molecular weight are in fact known to be able to efficiently cover the surface of colloidal nanoparticles, producing a valid stealth effect [13,14].

The length of the alkyl side chain in the LAA residue was also investigated: it is known that when the size of the lipid anchor matches the acyl chain length of the phospholipids in liposomal bilayers, a more stable incorporation of the PEG derivative can be attained [33]. Therefore, in the view of a more general use of these PEG–LAA conjugates, we chose LAA residues with a side alkyl chain ranging from 14 to 18 carbon atoms (m = 13–17 in Fig. 1), that might be spatially compatible with the most common phospholipids or fatty acids used to prepare liposomes or SLN (e.g., from myristic to stearic acid residues).

The synthesis of LAA conjugates with the α-methoxy-ω-carboxy-PEG_{5000} was easily achieved by a classical wet peptide-coupling procedure, in which a water soluble carbodiimide (EDAC) was used to simplify the work-out of the reaction mixture. After preliminary attempts, stoichiometric amounts of the starting carboxy-PEG_{5000} **1** and the wished LAA (as the methyl ester hydrochloride) were chosen for the reaction. The purification of crude products was made by semipreparative TLC. An analogous procedure was used to conjugate aminoα-methoxy-ω-amine-PEG_{5000} with the N-Boc-protected LAAs.

The products of the conjugation reaction of PEGs **1** and **3** with LAA-16, LAA-18, and LAA-20 were analyzed by MALDI-TOF MS. Here we discuss, as representative cases, the results obtained for the two conjugates with the LAA18 derivatives (compounds **2b** and **4b**).

Fig. 2a shows the MALDI mass spectrum of the starting carboxy-mPEG **1**: the spectrum is composed of peaks separated by 44 a.m.u., corresponding to the mass of the repeating unit of PEG (C_2H_4O). This polymer desorbs as sodium ion adduct (MNa^+). The inset displays a magnification of the central part of the spectrum, with the peak at m/z 5061.1 being the most intense signal and corresponding to MNa^+ ions with a degree of polymerization (DP) of 112. In the spectrum of the conjugate **2b** (Fig. 2b), the highest peak at m/z 5356.3 may be assigned to MNa^+ ions with a DP of 112, and no signals due to unreacted polymer are present. The mass difference between species with the same DP of 112 in the two spectra is 295.2, confirming the structures proposed in

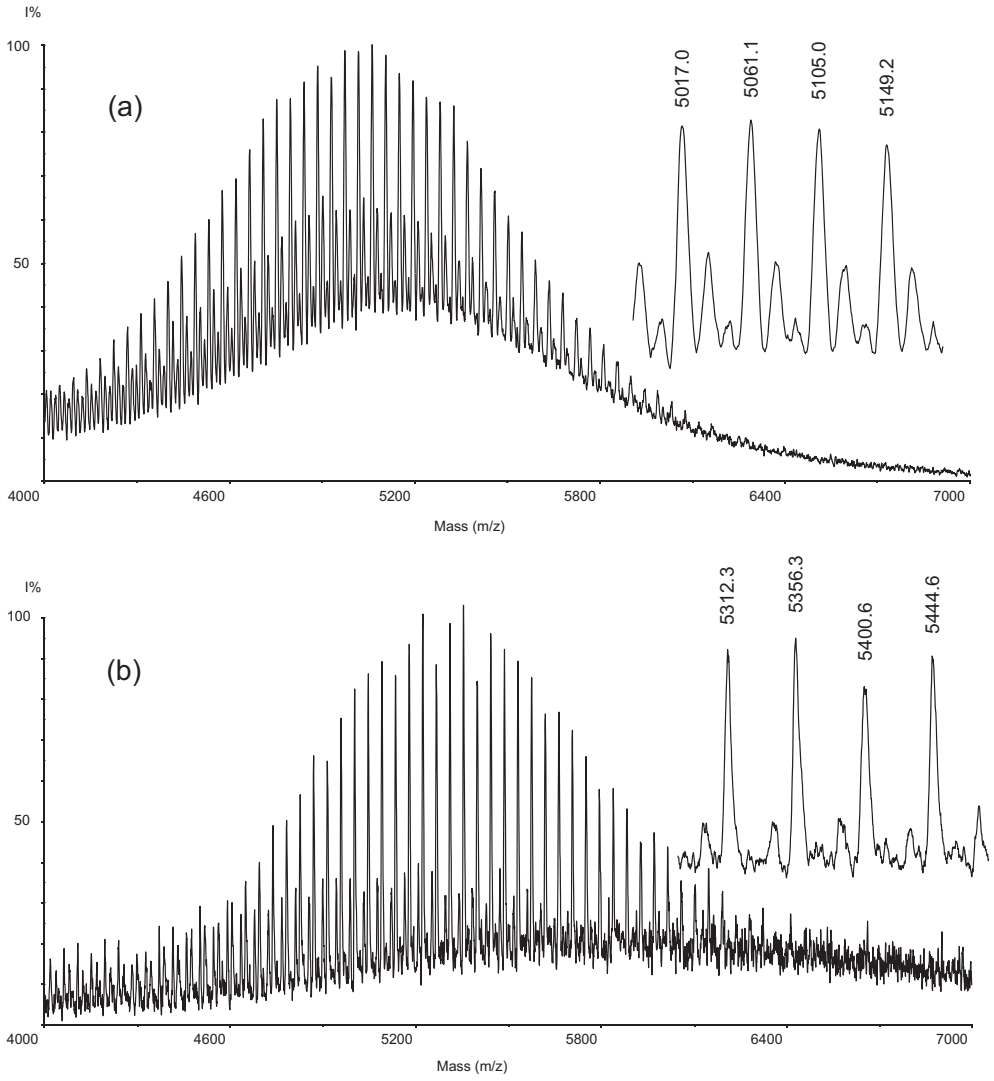


Fig. 2. MALDI mass spectrum of α-methoxy-ω-carboxy-PEG_{5000} 1 (a) and of its conjugate with LAA-18 **2b** (b).

Scheme 1. As the relative abundance of the various DP of the PEG molecules does not change in these spectra, the coupling reaction is not affected by the molecular mass of the PEG.

In the case of the starting amino-mPEG **3**, the MALDI mass spectrum of Fig. 3a shows that this PEG desorbs as protonated pseudomolecular ions (MH^+). The inset displays a magnification of the central part of the spectrum, with the peak at m/z 4569.2 being the most intense signal and corresponding MH^+ ions with a DP of 103. In Fig. 3b the spectrum of compound **4b** is shown. In this case, the most intense peak is found at m/z 4938.8, representing the MH^+ ions with DP of 105. The mass difference between species with the same DP in the two spectra, for instance the ion at m/z 4657.3 (DP = 105) in Fig. 3a and the ion at m/z 4938.8 in Fig. 3b is 281.5 a.m.u., which is the correct mass increment to be expected from the coupling reaction between the polymer **3** and LAA18. No peaks corresponding to the starting polymer were found in the spectrum of the conjugate **4b**. These data show that the structures obtained are those illustrated in Scheme 2 and that the coupling efficiency is independent on the DP.

The structure of the prepared conjugates was also confirmed by ^{1}H-NMR analysis, in which both signals of the starting PEG polymers and the conjugated LAA residue(s) were found (cf. Section 2.3). In particular, diagnostic peaks can be considered a triplet around 0.85 ppm, attributed to the terminal methyl group of the LAA alkyl chain, and signals around 1.2–1.3 ppm due to the methylene groups of the same moiety. At high resolution, a broad multiplet due to the α-CH hydrogen of the LAA residue was also observed around 4.5 ppm.

The formation of the amide bond between PEGs and LAAs in the conjugates was further corroborated by FT-IR spectroscopy, which showed the presence of amide I bands, around 1670 and 1650 cm^{-1}.

3.2. Solubility profile

The solubility of the synthesized conjugates was tested in a number of solvents of pharmaceutical interest with different polarity. In all the tested solvents the LAA conjugates of carboxy-PEG_{5000} (**2a–c**) and amino-PEG_{5000} with LAAs (**4a–c**) showed the same solubility pattern of the respective starting polymers **1** and **3**. The solubility at room temperature in water, as well as in the other tested solvents, of all the conjugates was higher than 100 mg/ml, i.e. more than 0.02 mol/l. As a comparison, in the same conditions the solubility of PEG 40 monostearate, frequently used for producing stealth lipid nanocarriers, was measured to be about 50 mg/ml (~0.025 mol/l) in water and ethanol, and higher than 200 mg/ml (>0.1 mol/l) in dichloromethane [15].

These data substantiate the amphiphilic character of the PEG_{5000}–LAA derivatives and, as already observed for the corresponding PEG_{2000}–LAA conjugates [15,16], indicate that the introduction of the LAA residue did not affect the solubility of the starting mPEGs. Such findings are also of particular interest in the view of their use for the production of stealth

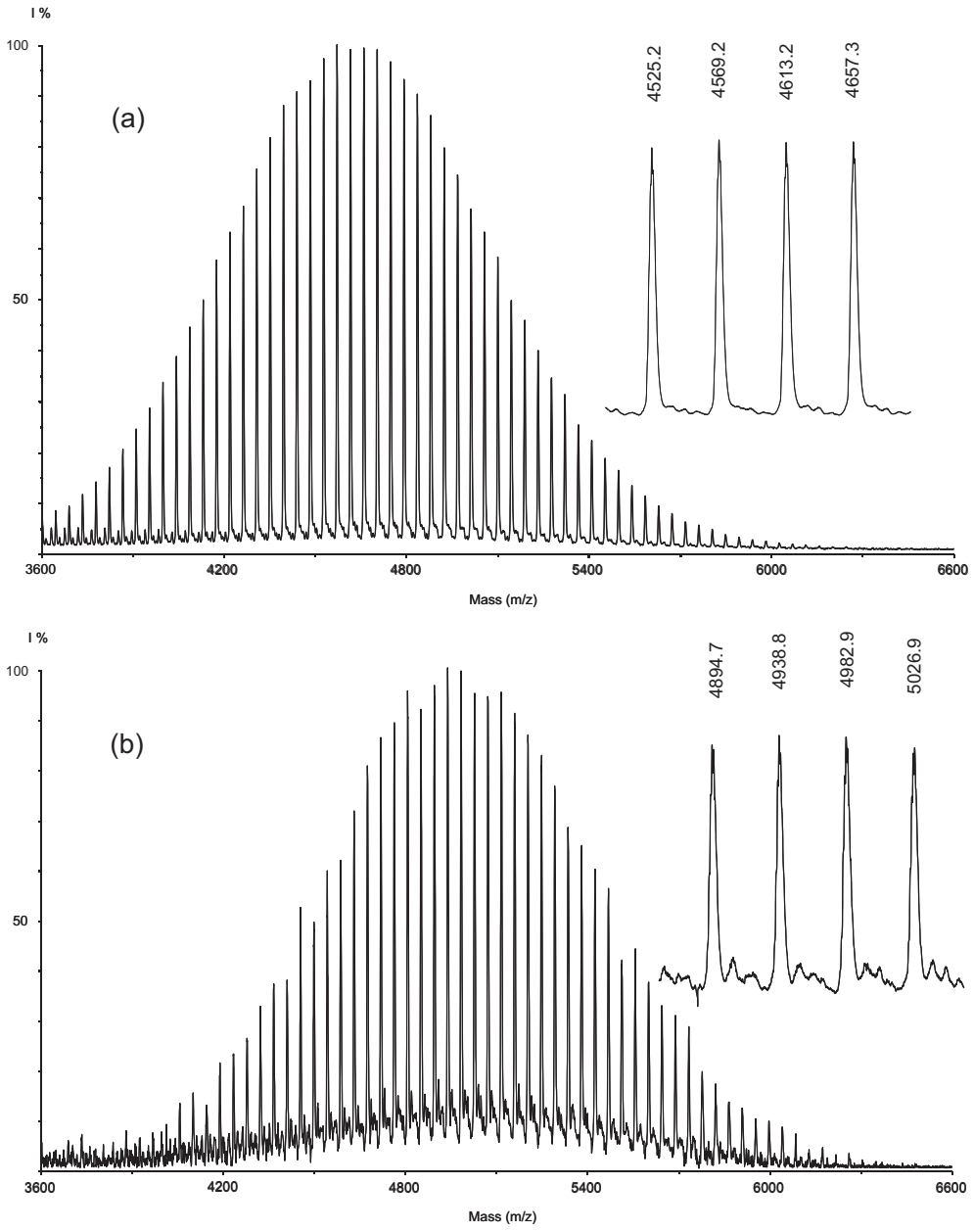


Fig. 3. MALDI mass spectrum of α-methoxy-ω-amino-PEG_{5000} 3 (a) and of its conjugate with LAA-18 **4b** (b).

nanoparticle carriers and liposomes, since the preparation of these systems often requires organic solvents, such as DCM, or aqueous solutions buffered at physiologically pH values.

3.3. Cytocompatibility assessment

The absence of cytotoxicity of the prepared conjugates was assessed by the MTT assay, a widely recognized in vitro preliminary screening able to individuate time- and concentration-dependent toxic effect on mammalian cell vitality and growth [33]. As shown in Fig. 4a, treatment of Caco-2 cell cultures with compounds **2b** and **4b**, representing the two synthetized series of PEG_{5000}–LAA conjugates, had no effect on cell viability, since, after 48 h of incubation, they did not reduce the ability of cells to metabolize the tetrazolium salt, compared to untreated control cells. A slight non-significant decrease in cell viability was only observed at the highest tested concentration (100 μM), that however corresponds to a much higher concentration of PEG derivatives (approximately 500 mg/l) than those used for the surface modification of liposomes or other nanocarriers. Therefore, these conjugates can be considered well tolerated by cells. However, detailed studies are in course to assess whether the initial signs of toxicity, observed at the higher doses, could be related to damaging effects on the DNA of the cells.

Mouse L929 fibroblasts were also used in this test as a prototype of normal cells. When they were incubated with the same conjugates **2b** and **4b**, no reduction of cell growth was observed (Fig. 4b), confirming the absence of cytotoxicity of the PEG–LAA derivatives.

3.4. DSC–biomembrane interaction studies

The covalent bond of LAA to PEGs would not only increase the lipophilicity of the starting polymers, but gave also an amphiphilic character to the final conjugates, a property associated, among others, to the interaction with biological and cell membranes [15,16,21,24,34].

We performed specific DSC studies to evaluate the interaction of the synthesized conjugates with a phospholipid bilayer model, consisting of MLV made of pure DPPC [35,36]. The obtained data could also furnish

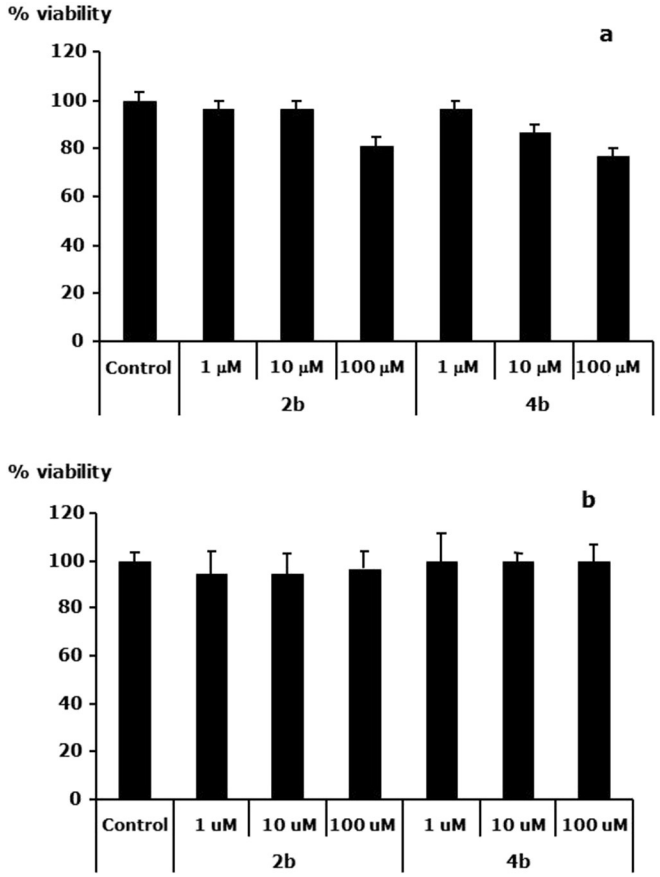


Fig. 4. Cell viability test (MTT assay) of Caco-2 cells (a) and L929 mouse fibroblasts (b) after 48 h of incubation with compounds **2b** or **4b**, at different concentrations.

useful information about the use of PEG–LAA as surface modifiers in the production of both liposomes and other colloidal drug carriers.

PEG_{5000}–LAA conjugates were added to DPPC, at the same concentrations at which other PEG–phospholipid derivatives, such as DSPE–PEG, are usually employed (1–10% in mol), during the preparation of the MLV. The consequent thermotropic behavior of the phospholipid bilayers were then studied by DSC. As reference calorimetric parameters, the variation of phase transition temperature (ΔT%) and of the associated enthalpy changes [Δ(ΔH)%], with respect to empty liposomes made of pure DPPC, were calculated.

The experimental findings, reported in Figs. 5–12 and in Tables S1–S8 (as Supplementary material), in general indicated that the addition of the polymer conjugates to MLV gave some effects upon the thermotropic profile of the DPPC bilayers, producing calorimetric evidences useful to understand their mode and depth of interaction. Firstly, disappearance of the endothermic pre-transition peak, typical of pure phospholipids (around 34.5 °C for DPPC), was always observed, even after the inclusion of the lowest molar concentrations of PEG_{5000}–LAA conjugates in the vesicles. The pre-transition peak corresponds to the transition from the Lβ phase to the Pβ ripple phase of the phospholipid bilayers, and is attributed to the rotation of the polar heads of phospholipids or to transformations in the structure of bilayers and changes in the hydrocarbon acyl chain packing within them [37]. This phenomenon is very sensitive to the presence of foreign molecules located in the polar region of the phospholipid bilayers [38]. Thus, the observed disappearance of pretransition, after the incorporation of conjugates in the DPPC vesicle dispersion, indicates that the compounds interact with the polar region of the bilayers and alter the tilting of DPPC acyl chains [39,40].

As expected, the addition of the pure starting polymers **1** (Fig. 5) and **3** (Fig. 9) did not affect the Tm value of DPPC bilayers, inducing only small changes compared to the calorimetric behavior of pure DPPC liposomes. The Tm value remained almost unchanged or slightly decreased with increasing the polymer mole fraction, and the reduction of

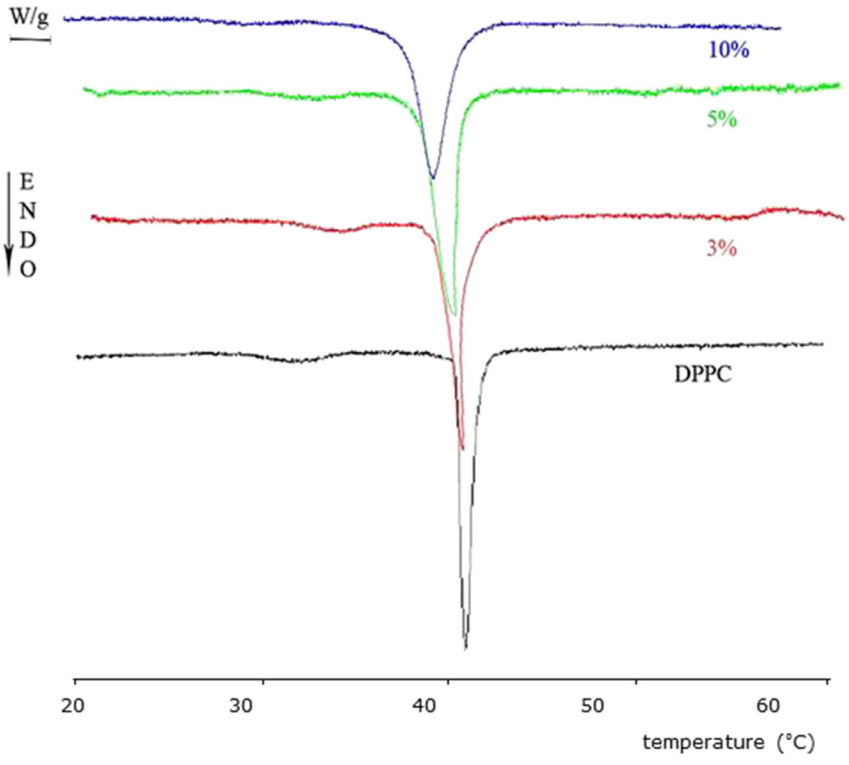


Fig. 5. DSC curves of the liposomes pure of DPPC or in the presence of increasing molar concentrations of **1**.

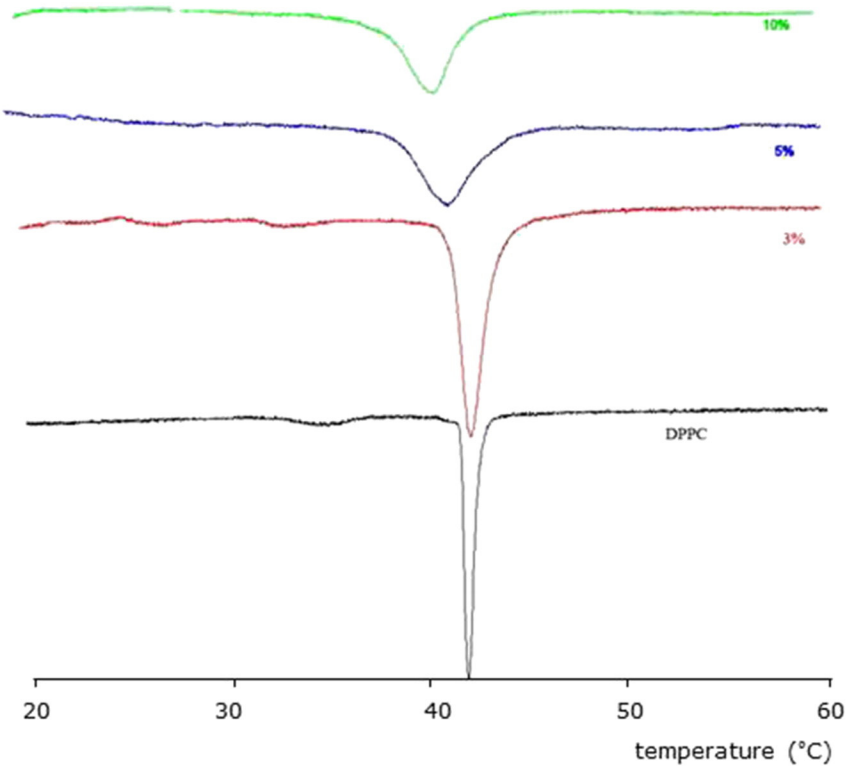


Fig. 6. DSC curves of the liposomes pure of DPPC or in the presence of increasing molar concentrations of **2a**.

enthalpy changes was small and inversely proportional to the initial molar concentration in the liposomal bilayers. This behavior was compatible with the hydrophilic nature of these polymers, and indicated that they were unable to localize deeply in the MLV structure, but affected only the more superficial phospholipid bilayers. Noteworthily, the reduction of Tm value caused by **1** and **3**, albeit limited, was greater than that previously measured for the corresponding carboxy- and amino-mPEG$_{2000}$ [15,16], in accordance with the greater hydrophilicity

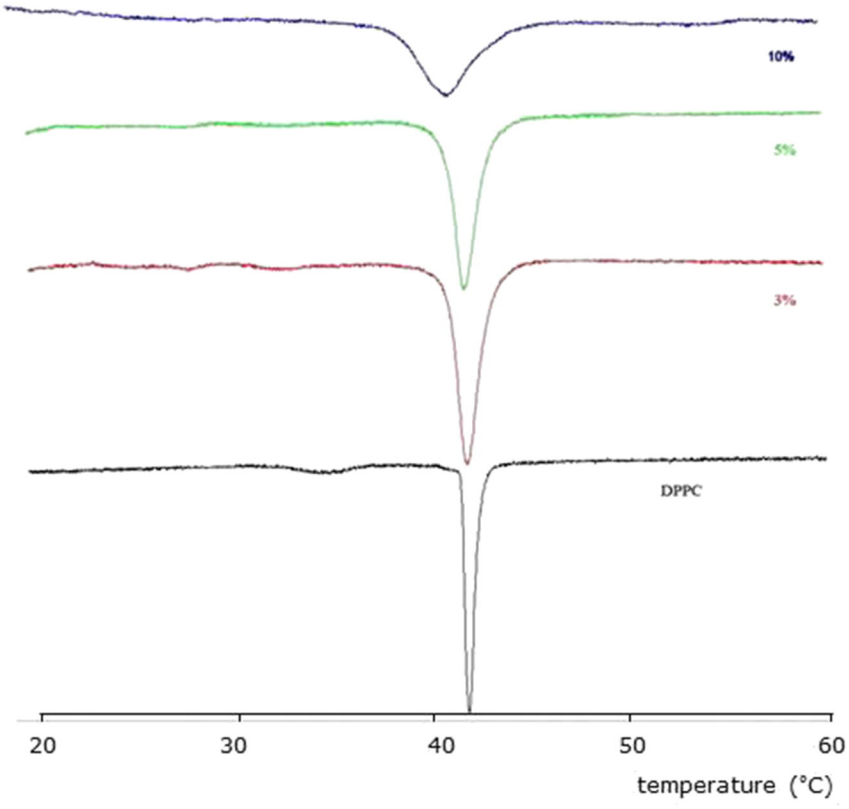


Fig. 7. DSC curves of the liposomes pure of DPPC or in the presence of increasing molar concentrations of **2b**.

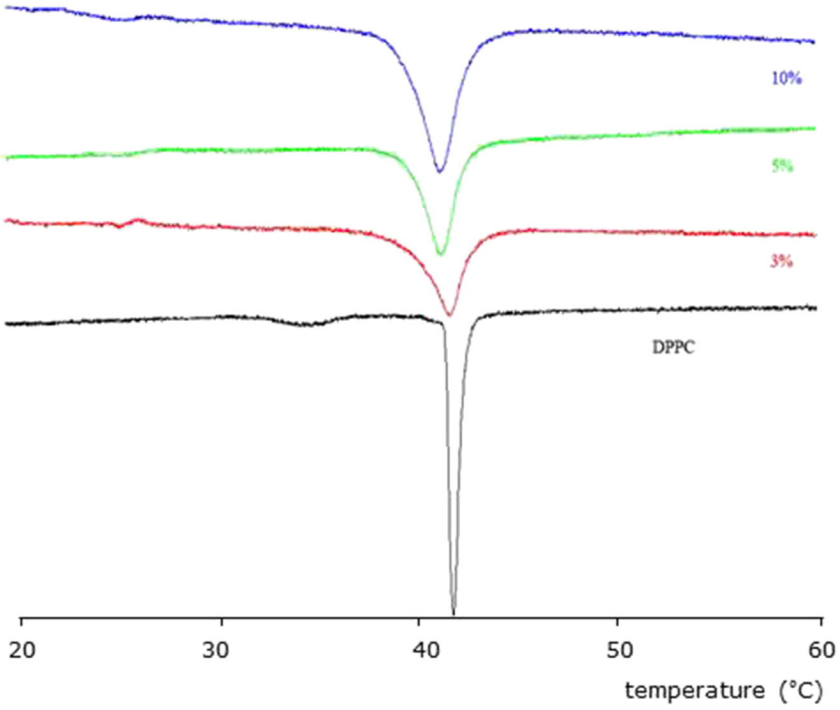


Fig. 8. DSC curves of the liposomes pure of DPPC or in the presence of increasing molar concentrations of **2c**.

density produced on the liposome surface by the higher molecular weight of these PEG_{5000} polymers.

The incorporation of increasing quantities (from 3 to 10% in moles) of the various PEG_{5000}–LAA conjugates in the MLVs, instead, induced noticeable effects upon the DSC profiles, compared to empty liposomes, related to the kind and amount of added conjugate. Such thermotropic effects were somehow predictable by considering the geometry of MLV prepared in the presence of the PEG–LAA conjugates: while the LAA alkyl chains were in fact interposed within the lipophilic domains (acyl chains) of DPPC bilayers, the PEG head remained outside or facing the interlamellar aqueous spaces. The latter hypothesis has also been demonstrated by us in other, yet unpublished experiments in which increasing concentrations of PEG–LAA conjugates were progressively able to shield the surface charge of negatively charged liposomes. As a

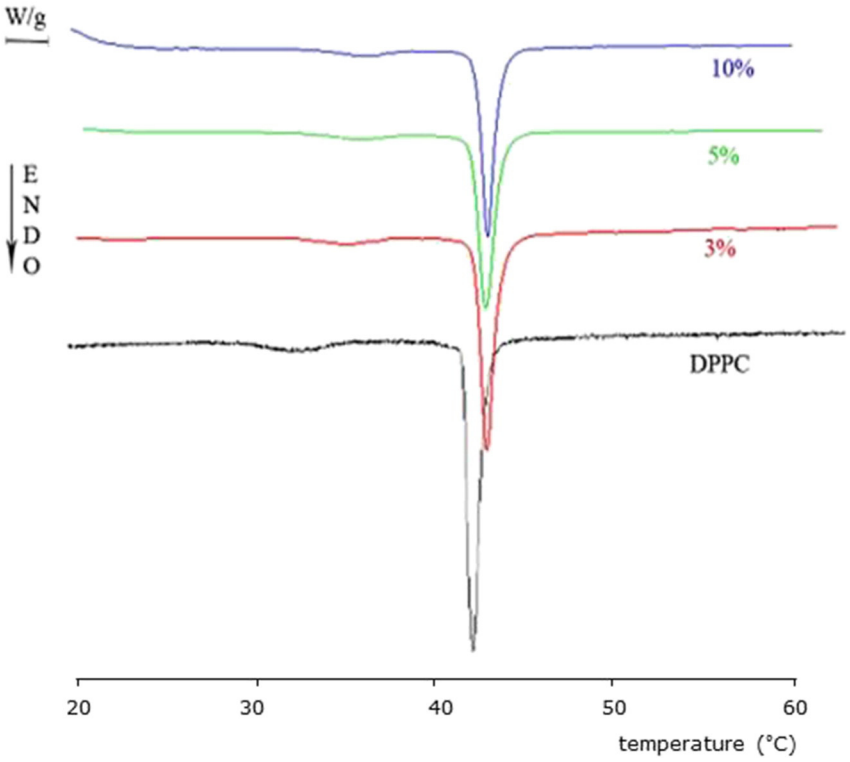


Fig. 9. DSC curves of the liposomes pure of DPPC or in the presence of increasing molar concentrations of **3**.

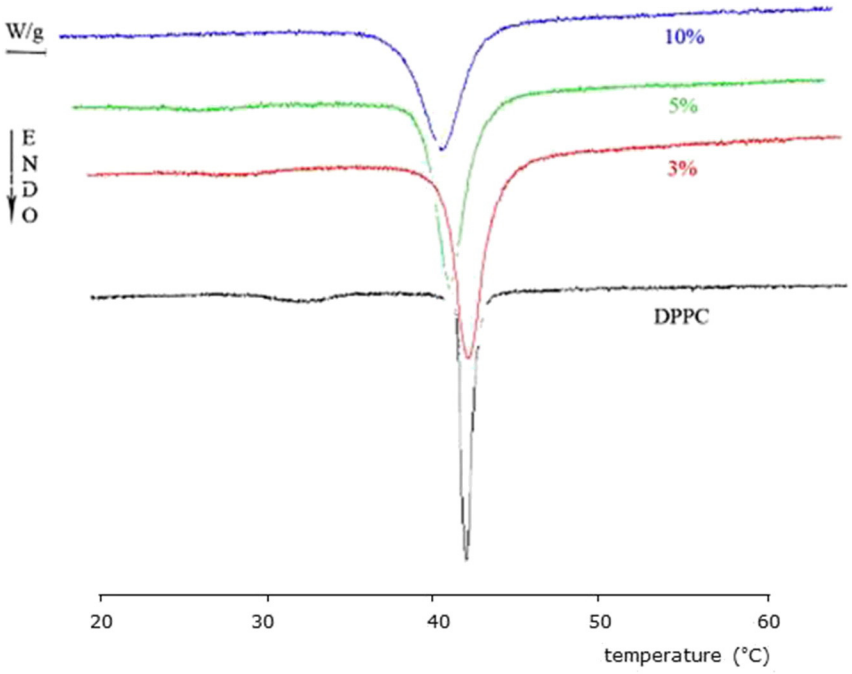


Fig. 10. DSC curves of the liposomes pure of DPPC or in the presence of increasing molar concentrations of **4a**.

consequence, a combined interaction occurred at both the levels of DPPC choline head groups and acyl chains.

The thermotropic data of compounds **2a–c** are shown in Figs. 6–8. The length of the aliphatic side chain of the LAA residue, conjugated to $mPEG_{5000}$-propionate, affected their degree of interaction with the liposomal bilayers. In particular, the lower homologous **2a** reduced the Tm value linearly with its initial concentration in the liposomes, coupled with a reduction of the ΔH and an increase of the phospholipid phase transition interval (Fig. 6). This was particularly evident at 5 and 10% molar concentrations. Taken together, these data suggest a more complex interaction of this conjugate with DPPC, than the starting PEG **1**, at both the levels of choline polar heads and acyl chains, and a greater penetration inside the internal bilayers, as demonstrated by the progressive enlargement of the endothermic phase transition peak (Fig. 6).

With the further lengthening of the aliphatic LAA side chain (conjugate **2b**), and thus of the lipophilicity of the conjugate, the effects on the 'cooperativity' of the liposomal systems were more evident, also at the lowest tested molar fraction (Fig. 7). Even more, the longest homologue **2c** induced a marked reduction of the ΔH value, combined with a limited lowering of the Tm value (Fig. 8). Such a behavior indicates that the latter conjugate, perhaps because of its high lipophilicity and limited aqueous solubility, was less able to disturb the packing of DPPC bilayers than its lower homologues **2a** and **2b**. Noteworthily, the corresponding derivative of carboxy-$mPEG_{2000}$ had shown to cause greater effects on DPPC bilayers [15], and this observation further support the concept that, with increasing the molecular weight of the hydrophilic PEG portion of these conjugates, their location on the used biomembrane model progressively became more superficial. This conclusion can be a

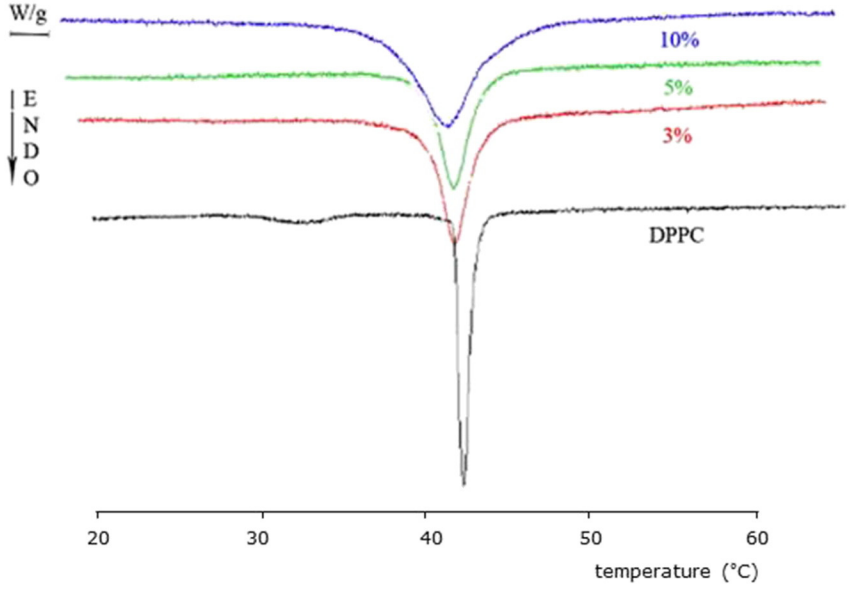


Fig. 11. DSC curves of the liposomes pure of DPPC or in the presence of increasing molar concentrations of **4b**.

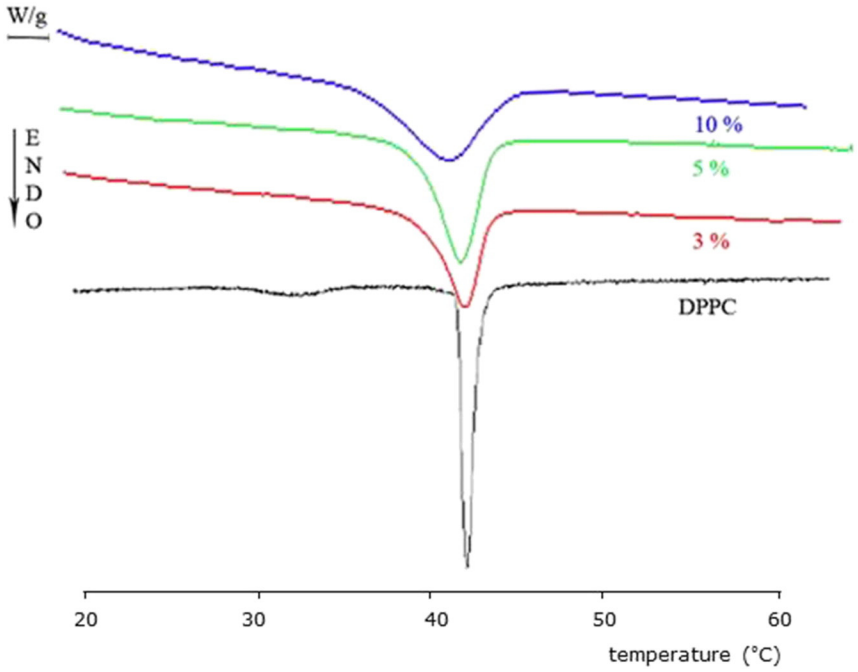


Fig. 12. DSC curves of the liposomes pure of DPPC or in the presence of increasing molar concentrations of **4c**.

positive aspect when considering their ability to cover the liposome or nanoparticle surface (PEGylation density) but, in the meantime, it can be viewed as a limitation of their PEGylation efficiency, since a scarce penetration inside the inner bilayers could result in an insufficient anchoring on the colloidal particles and, possibly, in a rapid removal (shedding) during storage or their circulation in the bloodstream.

Summarizing the overall DSC data registered for the carboxy-mPEG$_{5000}$–LAA conjugates **2a**–**c**, and confirming what observed for the corresponding carboxy-mPEG$_{2000}$–LAA conjugates [15], it can be outlined that the variations of the thermotropic parameters, compared to pure DPPC, were more marked at the 5% molar concentration, while a higher concentration was often associated to an incipient loss of homogeneity of the liposomal systems [15]. Such information can become useful in the forthcoming steps, when an optimal molar concentration of the PEG–LAAs must be chosen to prepare stable long circulating, drug-loaded nanocarriers.

For the sake of comparison, DPPC liposomes prepared in the presence of different percentages of DSPE–PEG (up to 15% in moles) did not show a significant change of the Tm value, but only a slight shape deformation of the endothermic peak of the phospholipid, with signs of an incipient phase segregation [15].

The calorimetric profiles of the MLV produced using the amino-mPEG$_{5000}$–LAA conjugates **4a**–**c** are shown in Figs. 9–12. As discussed before, the starting amino-mPEG$_{5000}$ **3** did not cause remarkable changes in the DSC profile of DPPC, nor in the shape of its endothermic main phase transition peak (Fig. 9). Conjugates **4a**–**c** instead interacted in a more complex way with the liposomal bilayers, with an evident relation to the length of the LAA side alkyl chain. In particular, the MLV modified with **4a** showed a reduction of the T_m value with increasing conjugate molar fraction (Fig. 10). Also the ΔH was affected, thus indicating a deep location of this compound inside the structure of the MLV. Also in this case, the lowering of the Tm value caused by **4a** was less marked compared to that one given by the corresponding amino-mPEG$_{2000}$ conjugate [16], since the larger hydrophilic portion in the former conjugate hindered its interaction with the phospholipid bilayers.

A similar thermotropic profile was shown by compound **4b** (Fig. 11), accompanied by a progressive broadening of the phase transition peak; these findings confirmed that also this conjugate, due to its amphiphilic nature, was able to interact not only with the external bilayers, but also with the inner parts of the MLV. The further elongation of the aliphatic side chain (compound **4c**) led to a small but linear reduction of the T_m value with increasing the conjugate molar fraction in the liposomes, associated to a decrease of the ΔH value (Fig. 12).

To summarize the DSC results relative to the amino-mPEG$_{5000}$–LAA conjugates **4**, it can be evidenced that the better interaction with the biomembrane model was given by the LAA16-derivative **4a**, up to a 5–10% molar concentration.

4. Conclusions

In this paper, two series of amphiphilic amide conjugates between carboxy- or amino-mPEG$_{5000}$ and LAA with a different side alkyl chain were prepared and characterized. By means of DSC, their interaction with a 3-D biomembrane model was studied, comparing their behavior with the parent hydrophilic PEG polymers. Contrarily to the starting (hydrophilic) polymers, the PEG–LAA conjugates, because of their amphiphilic nature, showed capacity to neatly arrange within the liposomal bilayers, with the aliphatic chains of the LAA residues interacting with the DPPC lipid domains and the hydrophilic PEG portion extruding on the external surface or in the aqueous inter-lamellar spaces of the vesicles.

Representative samples of the synthetized conjugates showed the absence of cytotoxicity on Caco-2 and L929 cell cultures.

This work belongs to a wider project aimed at obtaining alternative amphiphilic PEG derivatives than phospholipid-PEGs for the surface decoration of colloidal drug carriers. Therefore, in vitro experiments are in course and will be published lately to assess the ability of these PEG–LAA conjugates to efficiently cover the surface of liposomes and nanoparticles, by changing their surface properties and reducing uptake by phagocytic cells. The results should allow us to select the most promising conjugates for further in vivo studies, where the long-circulation properties and pharmacokinetics of colloidal particles containing these conjugates could be assessed.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2014.10.054.

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