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Application of thermal analysis to the study of lipidic prodrug incorporation into nanocarriers

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Special Chapter dedicated to the memory of dr. Michel Ollivon

Abstract Anti HIV molecules as numerous drugs cannot efficiently penetrate into the brain. Prodrug synthesis and encapsulation into pegylated nanocarriers have been proposed as an approach for brain delivery. Pegylated polymeric nanoparticles and liposomes were chosen to incorporate glycerolipidic prodrugs of didanosine. Differential scanning calorimetry experiments were performed on mixtures of prodrugs and lipids or polymer in order to study their interaction. The optimal incorporation ratios were determined for each prodrug and compared for both types of nanocarriers. All these results would be used to prepare optimised formulations of didanosine prodrugs loaded into pegylated nanocarriers for brain drug delivery.

Keywords Didanosine · DSC · Glycerolipidic prodrug · HIV · Incorporation ratio · Nanocarriers · Polyethyleneglycol

Introduction

Although numerous drugs are used to treat HIV infection with increasing efficacy, some tissues or cells like lymphatic macrophages or brain are infected by the virus and act as sanctuaries where drugs cannot penetrate and inhibit its replication. The design of new medicines able to reach these sanctuaries is actually a challenge. An approach based on prodrug synthesis and encapsulation into carriers has been proposed and applied to a nucleosidic analogue, the didanosine (ddI).

Both lipidic prodrugs of didanosine were developed in order to increase the passage of this drug through physiological membranes [1]. Didanosine is orally administered in the treatment of HIV infection and exhibits a relatively poor bioavailability (20–40%, [2]). Based on a mimetic approach, two glycerolipidic prodrugs of ddI were synthesized, a dipalmitoyl glycerol didanosine (ProddiNP) and a dipalmitoyl glycerol phosphate didanosine (ProddiP) (Scheme 1) [1]. The second prodrug was designed in order to avoid the limiting step of the first intracellular phosphorylation which limits the efficacy of didanosine on blocking the HIV replication into infected cells.

Previous studies combining HPLC and differential scanning calorimetry (DSC) analyses demonstrated the low solubility of both prodrugs into water and oils and their good incorporation into dipalmitoylphosphatidylcholine (DPPC) lamellae [3, 4]. Thermal analysis experiments were investigated to study the polymorphism of these amphiphilic molecules (Log P around 2), the influence of prodrug insertion on DPPC organization and to determine the maximal amount of each prodrug which could be incorporated into DPPC bilayers. Then prodrug loaded DPPC liposomes were prepared at the optimal ratios of prodrug/DPPC determined by DSC: 1/5 mol/mol for ProddiP and 1/10 mol/mol for ProddiNP. HPLC analyses showed good incorporation efficacies into liposomes loaded with ProddiP (93%) and ProddiNP (99%) [3, 4].

Both liposomal formulations of prodrugs demonstrated their ability to inhibit HIV replication into infected cells [1, 3, 4]. Moreover using a lipidic metabolism model, the

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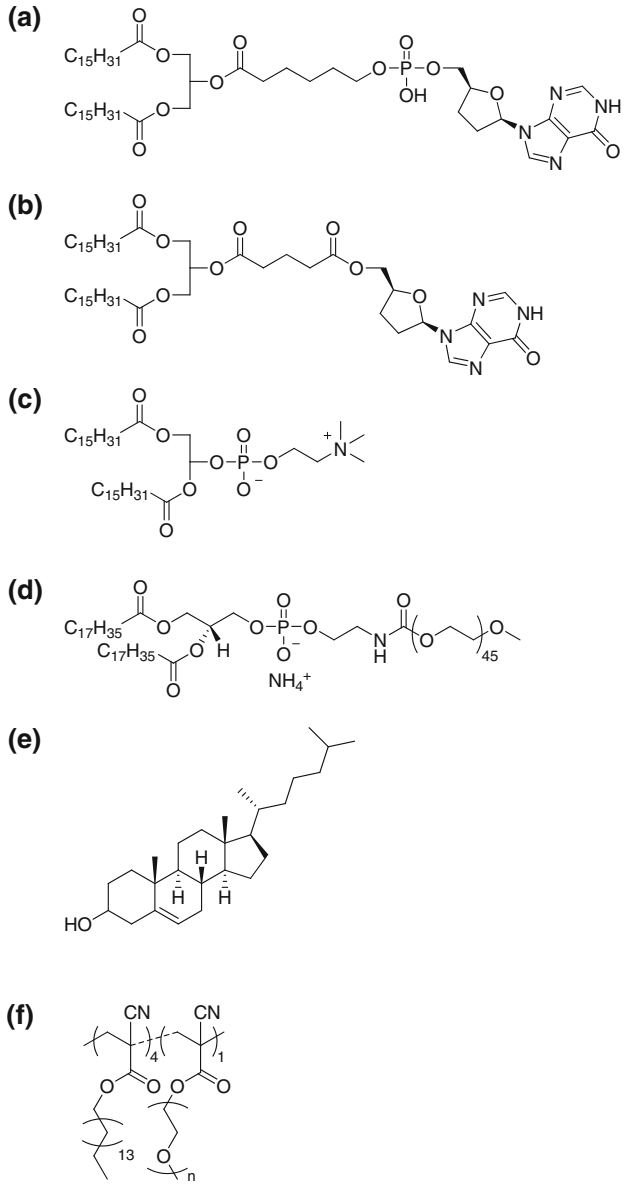

Scheme 1 Chemical structures of ProddiP (**a**), ProddiNP (**b**), DPPC (**c**), DSPE-PEG (**d**), Cholesterol (CHOL) (**e**) and PEG-PHDCA amphiphilic copolymer (**f**)

degradation of these glycerolipidic prodrugs by pancreatic lipases according to the metabolization scheme of long chain triglycerides was demonstrated [5]. This evidenced the structural analogy of our prodrugs with triglycerides. All these results suggested the capacity of these prodrugs loaded into lipidic formulations to enhance the penetration of ddI into intestinal membrane cells, to favor its localization into lymph where macrophages can be infected by HIV and to increase its oral bioavailability.

The localization of HIV into the brain is known to be responsible of associated dementia [6]. Drugs which are actually in the market are not efficient to avoid HIV infection into the patient brain. It can be explained by the fact that the blood brain barrier (BBB) is very efficient to limit the diffusion of hydrophilic drugs into the brain. A strategy is the design of lipophilic prodrug which can pass more through the BBB [7, 8]. Another approach is the encapsulation of drugs into a nanocarrier which can enter into the brain [8]. Recently, our team demonstrated that polymeric nanoparticles composed of poly[(hexadecyl cyanoacrylate)-*co*-methoxypoly(ethylene glycol) cyanoacrylate] copolymer (usually termed PEG-PHDCA) penetrated into the brain of animals after intravenous injection [9]. In vitro experiments demonstrated that these pegylated nanoparticles penetrated the BBB by an endocytosis mechanism [10]. The encapsulation of ddI prodrugs which are lipophilic molecules into pegylated nanocarriers seems a promising approach to increase ddI concentration into the brain and to treat HIV-1 associated dementia.

The aim of this study was to determine the pegylated nanocarriers capacity to incorporate didanosine glycerolipidic prodrugs. Numerous works have used DSC analyses in order to study the interactions between drug and excipients in order to determine their compatibility [11], their effect on drug dissolution [12] or release kinetic [13]. In this paper, DSC experiments were performed on both types of nanoparticles. First, ProddiP and ProddiNP encapsulations were studied into PEG-PHDCA nanoparticles. A second carrier, pegylated liposomes composed of 10/5/1 mol/mol/mol DPPC/cholesterol (CHOL)/distearoylphosphatidylethanolamine-polyethylene glycol (DSPE-PEG), were chosen for their long circulation time into the blood after intravenous injection [14]. DSC experiments were performed on prodrugs and lipids or copolymer mixtures in order to study these molecules insertion into lipidic bilayer or polymer matrix, respectively. The optimal incorporation ratios were determined for each prodrug and compared for both types of nanocarriers.

Materials and methods

Materials

Dipalmitoylphosphatidylcholine and cholesterol (CHOL) were provided by Sigma Aldrich (St Louis, USA). 1,2-Distearoyl-sn-glycerol-3-phosphatidylethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG) was obtained from Avanti Polar-Lipids (USA). Phosphate Buffer Solution (PBS) was provided by Gibco (Invitrogen, Cergy Pontoise, France). The prodrugs, ProddiNP and ProddiP, were synthesized as described in a previous study [1] by Artmolecules (Poitiers, France). Water was purified using a Synergy system from Millipore (France). The solvents were obtained from Carlo Erba (Rodena, Italy). The poly[(hexadecyl cyanoacrylate)-*co*-methoxypoly(ethylene glycol) cyanoacrylate] (PEG-PHDCA 1:4) copolymers

were synthesized by condensation of methoxy poly(ethylene glycol) cyanoacetate (MePEG, 2,000 Da) with hexadecyl cyanoacetate (HDCA) in ethanol, in presence of formalin and pyrrolidine as described elsewhere [9, 15, 16].

Methods

Preparation of prodrug mixtures with copolymer

ProddiNP Mixtures of ProddiNP and PEG-PHDCA copolymer at various prodrug/polymer ratios [5.3 and 10.0% (w/w)] were prepared by mixing adequate quantities of ProddiNP and copolymer in 0.2 mL of methanol and 0.1 mL of chloroform. Organic solvents were then evaporated under vacuum using a rotavapor, and the residual traces of organic solvents were evaporated under low vacuum. The powders obtained were directly used for DSC experiments.

ProddiP Mixtures of ProddiP and PEG-PHDCA copolymer at various prodrug/polymer ratios [5.2 and 11.4% (w/w)] were prepared by mixing adequate quantities of ProddiP and copolymer in 0.2 mL of chloroform. Organic solvents were then evaporated under vacuum using a rotavapor, and the residual traces of organic solvents were evaporated under low vacuum. The powders obtained were directly used for DSC experiments.

Preparation of prodrug mixtures with lipids

ProddiNP Mixtures of ProddiNP and DPPC/CHOL/DSPE-PEG 10/5/1 mol/mol/mol at various prodrug/lipids ratios [0/1, 1/40, 1/32, 1/24; 1/16, 1/8, 1/0 (mol:mol)] were prepared by mixing adequate quantities of ProddiNP and lipids in 0.2 mL of methanol and 0.1 mL of chloroform. Organic solvents were then evaporated under vacuum using a rotavapor, and the residual traces of organic solvents were evaporated under low vacuum. The lipidic film was then rehydrated with water with a 10:90 (w:w) lipid:water ratio.

ProddiP Mixtures of ProddiP and DPPC/CHOL/DSPE-PEG 10/5/1 mol/mol/mol at various prodrug/lipids ratios [0/1, 1/16, 1/8, 1/4.8; 1/3.2, 1/0 (mol:mol)] were prepared by mixing adequate quantities of ProddiP and lipids in 0.2 mL of chloroform. Organic solvents were then evaporated under vacuum using a rotavapor, and the residual traces of organic solvents were evaporated under low vacuum. The lipidic film was then rehydrated with water with a 10:90 (w:w) lipid:water ratio.

Differential scanning calorimetry (DSC) experiments

Thermal analyses were conducted by DSC, using a DSC-7 (Perkin-Elmer, St. Quentin en Yvelines, France). Samples were loaded in aluminium pans of 40 µL (pan, part no. BO14-3021 and cover, part no. BO14-3004, Perkin-Elmer) hermetically sealed. An empty, hermetically sealed aluminium pan was used as reference. Calibration was performed with indium (m.p. = 429.8 K, ΔHm = 28.45 J g^{-1}) and *n*-decane (m.p. = 243.6 K). The pan loading was performed at 293 K. The melting behaviour of the sample was monitored in the 273–363 K range at a scanning rate of 283 K min^{-1}. Baselines were performed before and after each group of analyses and indium was analysed each day in order to assure no variation of onset temperature and enthalpy superior to 0.1 °C and 0.1 J g^{-1}. These values have been determined from the DSC curves repeatability study which was performed on polymer and lipids samples without prodrugs in order to avoid modification of curves due to prodrug polymorphism.

Results and discussion

Usually the drug loading in formulation is determined by HPLC analyses after centrifugation of nanoparticles suspension. The low water solubility of prodrugs limits the efficacy to separate non loaded molecules which formed large crystals as observed by microscopy [3, 4] from carriers by centrifugation. This point evidenced the necessity to use another technique to study the incorporation of our prodrugs into lipids or polymer.

Both types of pegylated nanocarriers, polymeric and lipidic ones, were investigated for didanosine glycerolipidic prodrugs loading. Prodrugs mixtures with lipids (DPPC/CHOL/DSPE-PEG 10/5/1) or copolymer (PEG-PHDCA 1:4) were prepared and then studied by DSC analyses. The products chemical structures are presented in Scheme 1. A temperature range from 273 to 363 K and a heating rate of 283 K min^{-1} were used according to previous study [3, 4]. Moreover a loading temperature of 293 K was respected in order to avoid the melting of the prodrugs metastable forms before the registration. The aim of this study was to determine the highest prodrug/(lipids or polymer) ratio for which all the prodrug molecules were incorporated. DSC analyses could detect the presence of prodrug crystals by recording melting endotherms of prodrug.

Incorporation of prodrugs into PEG-PHDCA copolymer

The curve of PEG-PHDCA copolymer alone showed two main endothermic events at 306.2 and 319.8 K (Fig. 1). The thermal analyses of the PEG-PHDCA mixtures with increasing percentage of prodrugs aimed to study the insertion of each prodrug into the polymer by visualizing

modification of onset temperatures and enthalpies of the two endothermic peaks corresponding to the copolymer.

ProddiNP

The curve of ProddiNP exhibited two endotherms at 322.4 and 346.9 K separated by an exothermic peak around 336.2 K (Fig. 1a). Previous studies demonstrated the polymorphism of this molecule [4]. The first endotherm at 322.4 K corresponded to the ProddiNP metastable forms melting, the exothermic peak at 336.2 K to the recrystallization into more stable forms which melted at 346.9 K.

When ProddiNP was mixed with PEG-PHDCA at increasing percentage (Fig. 1a), it is to note the disappearance of the main endotherm peak at 346.9 K corresponding to ProddiNP. The two main endotherms corresponding to PEG-PHDCA were maintained with little modifications of onset temperatures. Only an endothermic peak at 328–329 K was observed but with very low enthalpy (<1 J g^{-1}) for both mixtures at 5.3 and 10.0% of ProddiNP which could correspond to the melting of ProddiNP metastable forms (Fig. 1b). In order to confirm the total incorporation of this prodrug in the polymer, the enthalpies corresponding to the two peaks of polymer melting were calculated as a function of the weight of polymer (J g^{-1} of PEG-PHDCA) in the sample (Table 1). The enthalpy decrease with the increasing percentage of prodrug suggested an increasing insertion of prodrug molecules into the crystalline matrix of the polymer which was not saturated until 10%.

All these results suggested that the optimum incorporation ratio of ProddiNP into PEG-PHDCA copolymer was above 10% w/w.

ProddiP

The ProddiP curve (Fig. 2) exhibited two main endotherms at 308.9 and 315.1 K corresponding to the melting of different polymorphic forms as observed in a previous study [3]. It is to note that these thermal events occurred in the same temperature range than those of PEG-PHDCA. The mixtures curves showed only two endotherms with onset temperatures closed to those observed for endotherms corresponding to the polymer and the prodrug melting.

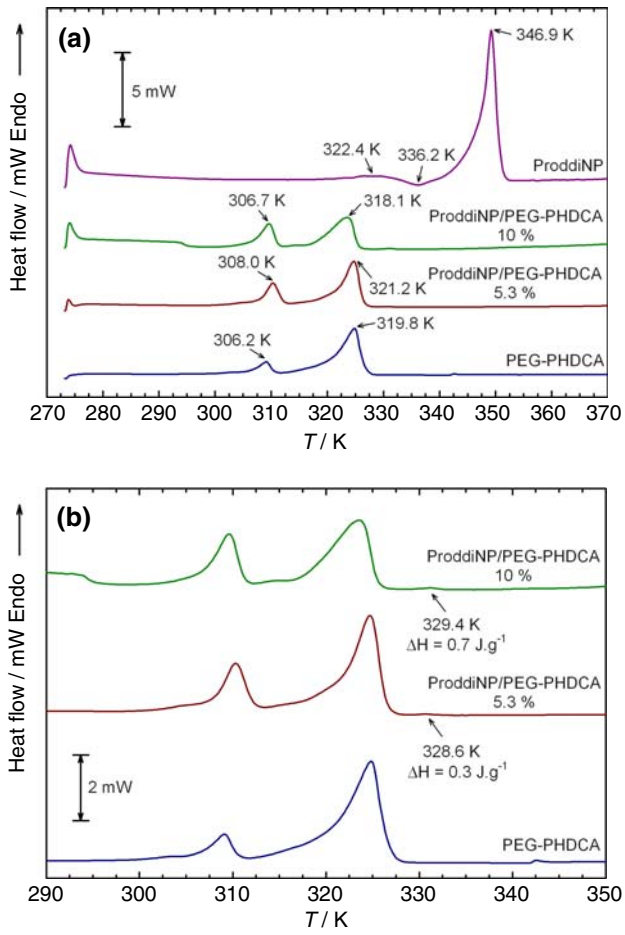

Fig. 1 **a** DSC recordings of ProddiNP/PEG-PHDCA mixtures at 5.3 and 10.0% (w/w) during their heating between 273 and 370 K at 283 K min^{-1}. **b** Details between 290 and 350 K

Table 1 Total enthalpy values of two main endotherms observed during the heating of ProddiNP/PEG-PHDCA mixtures calculated as J g^{-1} of polymer

Percent of ProddiNP into PEG-PHDCA mixtures (w/w) (%)	ΔH (J g^{-1} of polymer)
0 (polymer alone)	118.2
5.3	115.6
10.0	110.3

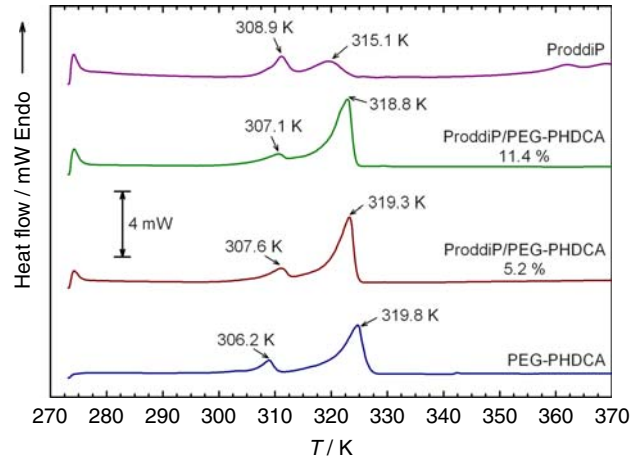

Fig. 2 DSC recordings of ProddiP/PEG-PHDCA mixtures at 5.2 and 11.4% (w/w) during their heating between 273 and 370 K at 283 K min^{-1}

Table 2 Total enthalpy values of two main endotherms observed during the heating of ProddiP/PEG-PHDCA mixtures calculated as J g^{-1} of polymer

Percent of ProddiP into PEG-PHDCA mixtures (w/w) (%)	ΔH (J g^{-1} of polymer)
0 (polymer alone)	118.2
5.2	134.1
11.4	147.8

Table 2 presents the total enthalpies of the two observed peaks calculated as a function of the polymer content. In the case of both mixtures, higher enthalpies (increase >15 J g^{-1}) were found than for the polymer alone suggesting that a part of prodrug was not incorporated into the polymer, even at the lower percentage of ProddiP. The optimum incorporation ratio into PEG-PHDCA seemed to be under 5% w/w of ProddiP.

Incorporation ratio comparison of both prodrugs into polymer

Differential scanning calorimetry analyses allowed determining the higher amount of each didanosine prodrug which could be incorporated into the PEG-PHDCA polymer: >10 and <5% w/w for ProddiNP and ProddiP, respectively. PEG-PHDCA is an amphiphilic copolymer composed of an hydrophilic part, namely methoxy-poly(ethylene glycol) cyanoacrylate and an hydrophobic part, namely poly(hexadecyl cyanoacrylate) (Scheme 1). It is well-known that the synthesis provides a complex mixture of various oligomers exhibiting different amphiphilic properties centered on 1:4 (hydrophilic:hydrophobic) molar ratio [17]. This ratio indicated that the formed copolymer were more hydrophobic than hydrophilic. The incorporation of the amphiphilic prodrugs into these polymeric mixtures seemed to be influenced by the lipophilicity of these molecules. The ProddiNP without the phosphate group was more hydrophobic and in consequence significantly more (at least ×2) incorporated into this polymer.

Incorporation of prodrugs into lipidic mixtures

ProddiNP

No thermal events were observed in the curve of the lipidic mixture composed of DPPC/CHOL/DSPE-PEG 10/5/1 mol/mol/mol (Fig. 3). When pure lipids were analyzed in the same conditions, their curves revealed a main endotherm at 314.2 K for DPPC, one endotherm at 351.2 K for CHOL and no thermal event for DSPE-PEG (data not shown). DSC analysis of ternary mixture evidenced the interaction between the three lipids.

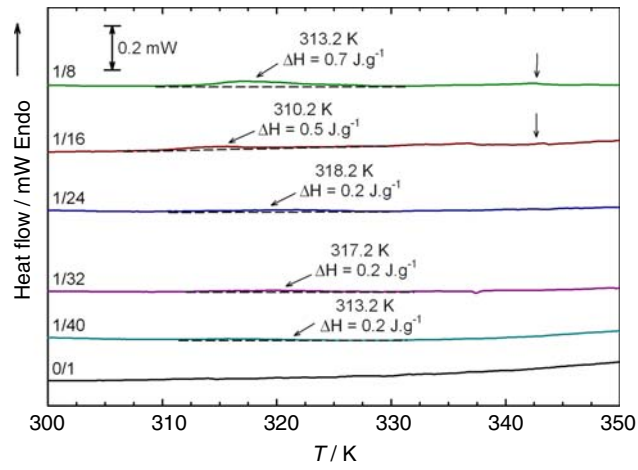

Fig. 3 DSC recordings of ProddiNP/lipids mixtures at various molar ratios (from 0/1 to 1/8) during their heating between 300 and 350 K at 283 K min^{-1}

When ProddiNP was added to the lipidic mixture, only endotherms of low enthalpies could be observed (Fig. 3). The main one with an onset temperature between 310.2 and 318.2 K seemed to correspond to the DPPC melting. It is to note that this peak was very difficult to determine because of a very low enthalpy of 0.2 J g^{-1} which was constant for 1/40, 1/32 and 1/24 ProddiNP/lipids molar ratios. Moreover no peak at temperature above 333.2 K corresponding to the prodrug melting was observed for these ratios. The prodrug seemed to be completely incorporated at these ratios. For ratios above 1/24, the curves exhibited a main endotherm around 313.2 K with increasing enthalpy and a little peak around 343.2 K seemed to appear (arrows in inset of Fig. 3). These data suggested that a little part of ProddiNP was not incorporated into the lipidic mixtures for 1/16 and 1/8 ProddiNP/lipids molar ratios. At these higher ratios, there was probably a coexistence of lipid bilayers saturated in ProddiNP and small crystals of prodrug perhaps stabilized by lipids. Therefore, the optimal incorporation ratio for this prodrug into DPPC/CHOL/DSPE-PEG mixtures was supposed to be between 1/16 and 1/24 mol/mol.

ProddiP

As for ProddiNP, the curves of the phosphated prodrug and lipids mixtures showed only endotherms of low amplitude (Fig. 4). For 1/16 and 1/8 molar ratios of ProddiP/lipids, the endotherm observed at 312–313 K could be attributed to the melting of DPPC chains. At 1/5 and 1/3 molar ratios, the onset of the main endotherm was displaced towards 308.2 and 306.2 K, respectively which corresponded to the melting temperature of ProddiP. Moreover the enthalpy of these peaks increased above 1 J g^{-1}. ProddiP seemed to be

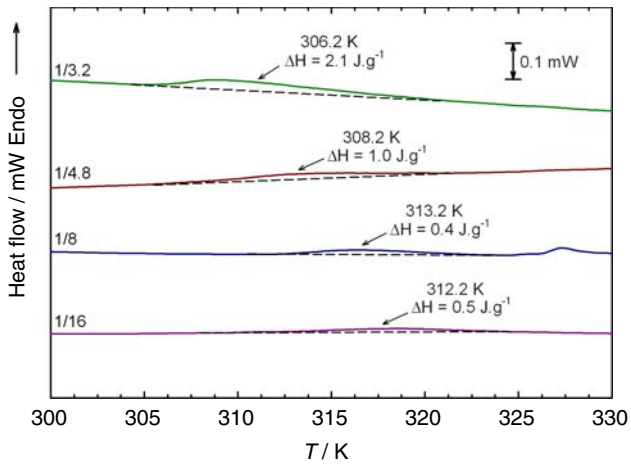

Fig. 4 DSC recordings of ProddiP/lipids mixtures at various molar ratios (from 1/16 to 1/3.2) during their heating between 300 and 330 K at 283 K min^{-1}

not completely incorporated at these ratios. These results suggested that the optimal incorporation ratio of ProddiP into DPPC/CHOL/DSPE-PEG mixtures was between 1/8 and 1/5 mol/mol.

Comparison of incorporation ratio of both prodrugs into lipids

The optimal incorporation ratios of ProddiNP and ProddiP into lipidic mixtures were compared with previous results determined by thermal analysis in the same conditions (temperature range and heating rate) [3, 4] (Table 3). We can note an influence of lipid nature on the incorporation of prodrugs. For ProddiP which has a greater structural analogy with phospholipid than the other prodrug (Scheme 1), the incorporation was increased by replacing DPPC by EPC. EPC is a mixture of phospholipids with acyl chains of various lengths and in liquid state which can easily integrated a larger amount of amphiphilic molecules than DPPC as observed in previous experiments with surfactants [18–20]. This difference is not observed in the case of ProddiNP for which the optimal incorporation ratio was the same in EPC or DPPC lamellae. These data suggested a difference in the localisation of each prodrug molecule into the phospholipidic bilayer. This hypothesis was confirmed by the difference of DPPC molecular organization (d_{001} = 64 Å, d = 4.2 Å) in presence of ProddiP (d_{001} = 74 Å, d = 4.14 Å) or ProddiNP (d_{001} = 71 Å, d = 4.23 Å) as measured by small angle and wide angle X ray diffraction experiments, respectively [4]. These data suggested the localizations of ProddiP probable nearer DPPC head groups and ProddiNP more between DPPC acyl chains. Moreover the higher sensibility to acidity of ProddiP (62% of degradation after 30 min incubation at pH 1, personal data) than ProddiNP (56%, [4]) in DPPC liposomes suggested also a deeper insertion of ProddiNP into the phospholipidic bilayers.

Table 3 Comparison of incorporation ratios of ProddiP and ProddiNP into various lipidic mixtures determined by DSC analyses

Lipids	ProddiNP/Lipids ratio (mol/mol)	ProddiP/Lipids ratio (mol/mol)
EPC	1/10[a]	1/3[a]
DPPC	1/10[a]	1/5[b]
DPPC/CHOL/DSPE-PEG 10/5/1 (mol/mol/mol)	1/16 to 1/24	1/5 to 1/8

Data from [4][a] and [3][b]

The ProddiP incorporation ratios into DPPC and DPPC/CHOL/DSPE-PEG mixture were similar or just a little decreased in the mixture (Table 3). At the opposite, the ProddiNP incorporation ratio was significantly decreased in lipid mixtures in comparison with DPPC alone. It could be explained by the fact that CHOL presence clearly modified the DPPC organisation as indicated by the DPPC melting endotherm disappearance in curve of DPPC/CHOL 10/5 (mol/mol) mixture (data not shown). CHOL molecules were inserted between DPPC acyl chains. DPPC/DSPE-PEG 10/1 (mol/mol) mixtures curve (data not shown) exhibited only a weak decrease of melting enthalpy without change of DPPC melting temperature suggesting a slight DPPC bilayers disorganisation. When ProddiNP/lipids ratio increased, DPPC/CHOL/DSPE-PEG bilayers were saturated in ProddiNP molecules for lower ratio than DPPC ones due to a probable competition between CHOL, DSPE-PEG and prodrug molecules for insertion between DPPC molecules. Because ProddiP molecules were more localized at the surface of DPPC and DPPC/CHOL/DSPE-PEG bilayers, their incorporation was less influenced by the DPPC disorganization induced by CHOL and DSPE-PEG insertion.

Comparison of incorporation ratio of both prodrugs into polymer and lipids

The Table 4 resumes the optimal didanosine prodrug incorporation ratios into DPPC/CHOL/DSPE-PEG mixtures and in PEG-PHDCA copolymer. The ProddiP incorporation was better in lipidic mixtures (16.2–26.0%) than

Table 4 Comparison of incorporation ratios of didanosine prodrugs into lipids and polymer

Excipients	ProddiNP/excipients (w/w) (%)	ProddiP/excipients (w/w) (%)
DPPC/CHOL/DSPE-PEG	5.0–7.5	16.2–26.0
PEG-PHDCA	10.0	<5.2

in copolymer (<5.2%). This high difference could be attributed to the exclusion from the hydrophobic polymer of ProddiP which was more hydrophilic. Moreover, the phosphated prodrug structural analogy with phospholipids and especially DPPC could explain its high capacity of mixing. At the opposite ProddiNP incorporation was higher in polymer (10%) than in lipids (5.0–7.5%) and the difference was less dramatic. Similarly, these results could be attributed to the increased ProddiNP lipophilicity and its lower structural analogy with phospholipids. But the difference between incorporations into polymer and lipids was smaller thanks to its amphiphilic nature which probably reduced its incorporation in polymer and increased its mixing into lipids in comparison with more hydrophobic compounds.

Conclusions

Differential scanning calorimetry experiments were performed in order to study both diglyceridic didanosine prodrugs interaction with PEG-PHDCA copolymer and DPPC/CHOL/DSPE-PEG mixture. The best incorporation ratios of each prodrug into polymeric matrix and lipidic mixtures were determined and compared evidencing the influence of their lipophilicity and structural analogy with DPPC. Moreover these data coupled with previous data obtained by DSC, X ray diffraction and degradation in acidic medium allowed to evidence a difference of prodrug localizations into lipidic bilayers. All these results will be used to prepare optimised and specific formulations of didanosine prodrugs loaded into pegylated nanocarriers for brain drug delivery.

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