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Research paper

Development and physico-chemical characterization of a liposomal formulation of istaroxime

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ABSTRACT

Istaroxime, an investigational new drug that targets defective Ca^{2+} cycling without compromising cardiac efficiency, may represent a promising and safe treatment of both acute and chronic heart failure. Even though the compound demonstrated good tolerability in a phase I/II safety study, symptoms related to the gastro-intestinal tract and pain at the injection site were reported as the most frequent side effects. The aim of this study was to encapsulate istaroxime in a drug delivery system (DDS) that could minimize the pain perceived upon administration. The DDS was designed to be quickly destabilized in plasma, in order to minimize alteration of the pharmacokinetic profile of istaroxime. To meet those requirements, a balance between the encapsulation efficiency and the release rate was sought. Transmembrane pH-gradient liposomes formulated with different phosphatidylcholines were investigated as vehicles for an efficient active drug loading. Poly(ethylene glycol)–660-hydroxystearate (PEG–HS) was chosen as excipient to modulate the bilayer fluidity and the release properties of the liposomes. A fast and efficient encapsulation was obtained by modulating the drug-to-lipid ratio, the amount of PEG–HS, and the incubation temperature. High encapsulation efficiency was achieved by incubating the drug with liposomal dispersions at room temperature for 10 min. Almost complete release was obtained in physiological conditions in less than 10 min, suggesting a model formulation potentially useful for drugs presenting similar features and side effects.

1. Introduction

Heart failure (HF) afflicts over 5 million Americans and 15 million Europeans and Mediterraneans [1,2]. It represents a serious public health burden, associated with major morbidity and mortality. It has been described as the "emerging" epidemic for the 21st century: among all adults aged 40 years, 1 in 5 will develop heart failure at some point in his/her lifetime [3]. Since the introduction of digoxin into clinical practice, inotropic agents have played a pivotal role in HF treatment. However, their proarrhythmic potential has led to a radical reassessment of their use for this indication [4]. Agents with innovative mechanisms of action are currently being tested, and further improvements are ongoing [5,6].

Istaroxime (Fig. 1) is a novel Na^+/K^+-ATPase inhibitor that has been shown to have the unique property of increasing sarcoplasmic reticular (SR) calcium adenosine triphosphatase isoform 2a (SERCA2a) activity [7,8]. By inhibiting Na^+/K^+-ATPase activity, a cytosolic calcium accumulation is induced (inotropism) while, due to SERCA2a stimulation, subsequent SR calcium storage is increased, preventing Ca^{2+} intoxication and facilitating myocardial relaxation (lusitropism) [9]. By exerting both inotropic and lusitropic activities, istaroxime targets defective Ca^{2+} cycling without compromising cardiac efficiency, representing an effective and safe treatment of both acute and chronic HF [10]. Animal models have shown that istaroxime promotes both muscle contraction and relaxation and prevents the cytosolic calcium overload and arrhythmia associated with digoxin [9]. The compound demonstrated good tolerability in a phase I/II safety study. However, unexpected symptoms related to the gastro-intestinal (GI) tract and pain at the injection site were reported as most frequent side effects [11,12]. Pain upon injection represents a serious drawback for parenteral administration. Such a problem could be solved by reducing the interaction of the drug with the surrounding tissues at the injection site through encapsulation in suitable formulations [13].

In choosing an appropriate drug delivery system for istaroxime, several aspects should be considered: (a) a final dosage form that

Abbreviations: DPPC, 1,2-myristoyl-sn-glycero-3-phosphocholine; DMPC, 1,2-palmitoyl-sn-glycero-3-phosphocholine; DOPC, 1,2-oleoyl-sn-glycero-3-phosphocholine; EPC, egg phosphocholine; PEG–HS, poly(ethyleneglycol)–660-hydroxystearate; HPTS, 1-hydroxypyrene-3,6,8-trisulfonic acid; DPX, p-xylenebis(pyridinium bromide; FBS, fetal bovine serum; Ch-BODIPY, cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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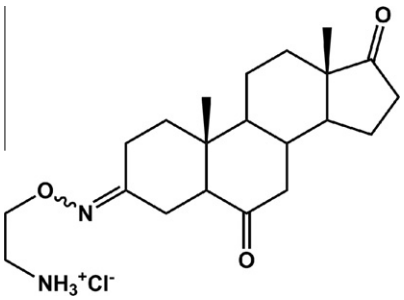

Fig. 1. Chemical structure of istaroxime.

can be easily stored and reconstituted; (b) the presence of a biocompatible barrier able to interfere with the pain perceived at the injection site; and (c) rapid destabilization of liposomes after injection to minimize alteration of the drug pharmacokinetic profile. Liposomes appeared to be an ideal choice to overcome the drawbacks presented by the current administration route of istaroxime, providing versatility in terms of formulation composition and drug-loading procedures [13]. The chemical properties of istaroxime played a crucial role in selecting the most appropriate loading procedure. Istaroxime is an amphipathic weak base (pK_a 9.25) that could, in principle, be remotely loaded within large unilamellar vesicles (LUVs) exhibiting a transmembrane pH gradient [14,15]. The good stability of the drug in its dry form and its relative chemical lability in aqueous media led to the choice of adopting an active *in situ* loading procedure [16]. The drug would therefore be stored as a powder, reconstituted, and loaded into the liposomes just prior to the intravenous infusion. Drug release should occur rapidly after injection to avoid a drastic change in the pharmacokinetic profile of istaroxime.

Liposomal membrane permeability can be adapted by varying the phospholipid composition and by incorporation of additives, such as surfactants. Phosphatidylcholines (PC) with different chain lengths and levels of saturation were chosen as main components of the bilayer. In order to keep the formulation as simple as possible, permeability of the bilayer was modulated by the addition of a well-tolerated surfactant, poly(ethylene glycol)–660-hydroxystearate (PEG–HS). This excipient was chosen for its low toxicity profile, its good tolerance upon parenteral administration, and its compatibility with the lipid bilayer. Parameters such as drug-to-lipid ratio, PEG–HS concentration, and incubation temperature were varied, and their effect on encapsulation efficiency and release kinetics were investigated.

2. Materials and methods

2.1. Chemicals

Istaroxime was provided by Debiopharm S.A. (Gland, Switzerland). 1,2-myristoyl-*sn*-glycero-3-phosphocholine (DMPC),1,2-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-oleoyl-*sn*-glycero-3-phosphocholine (DOPC), and egg phosphocholine (EPC) were a gift from Lipoid (Ludwigshafen, Germany). Poly(ethylene glycol)–660-hydroxystearate (PEG–HS) was kindly donated by BASF (Friedrichshafen, Germany). 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS), *p*-xylenebis(pyridinium bromide) (DPX), Gibco fetal bovine serum (FBS), and cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoate (Ch-BODIPY) were obtained from Invitrogen (Eugene, OR). All the other chemicals were purchased from Sigma Aldrich (St. Louis, MO). The solvents used in the serum extraction protocol were HPLC grade. Water was distilled and deionized in a Barnstead NANOpure Diamond Ultrapure Water Systems (Thermo Scientific, Rockford, IL). The osmolalities of the solutions used were measured with a Wescor 5500 vapor pressure osmometer (Wescor, Logan, UT) and eventually adjusted with NaCl to obtain isotonicity.

2.2. Liposome preparation

Lipid vesicles containing the selected phosphatidylcholines (DOPC, EPC, DMPC, or DPPC, unless otherwise stated) mixed with 0, 5, 10, or 15 mol% PEG–HS were prepared according to the film hydration extrusion method [17]. Briefly, stock solutions of lipids in $CHCl_3$ and a stock solution of PEG–HS in ethanol were mixed in order to obtain the chosen molar percentage of surfactant. Organic solvents were evaporated under a stream of nitrogen until dry. Traces of solvent were further removed by keeping the lipid films under vacuum overnight. The swelling buffer and the procedures followed to obtain LUV or multilamellar vesicles (MLV) are described in more detail in the Supplementary Information. Unless otherwise stated, the total lipid concentration was 5 mM.

2.3. Dynamic light scattering (DLS)

The mean diameter and size distribution of liposomes was measured by DLS at a scattering angle of 165° using a Delsa Nano S instrument (Beckman Coulter, Krefeld, Germany) equipped with a 658-nm laser diode and a temperature controller. The intensity size distributions of the liposomes were typically unimodal, and therefore, the autocorrelation functions were analyzed according to the cumulant method.

2.4. Differential scanning calorimetry (DSC)

DSC measurements were performed using a DSC Q200 (TA Instruments, New Castle, DE) and the data analyzed with Q Series software. Temperature and enthalpy calibration was performed using indium as reference. About 10 μL of liposome solution was transferred to an aluminum pan and hermetically sealed. The reference pan was filled with the appropriate buffer solution. The weight of each DSC pan was verified before and after the temperature scan to check for eventual water leakage. The scan rate was 2 °C/min. After an initial isothermal period of 5 min, the MLV were scanned between the temperature ranges reported in Supplementary Table S1.

2.5. Fluorescence spectroscopy

2.5.1. Steady-state fluorescence polarization

The liposome dispersions (EPC, DOPC, DMPC, or DPPC) were diluted into quartz cuvettes (final concentration 0.1 mM), and 1,6-diphenyl-1,3,5-hexatriene (DPH) from a THF stock solution was added (probe/lipid molar ratio 1:300). The dispersions were stirred and incubated at a temperature above T_m until fluorescence intensity reached a plateau. The eventual presence of free DPH in solution was checked by recording the fluorescence after addition of a small excess of liposomal suspension [18]. The change in polarization as a function of temperature and surfactant concentration was measured by means of a Cary Eclipse Fluorescence spectrophotometer (Varian Inc., Palo Alto, CA) equipped with manual polarizers and a Peltier circulating water bath to strictly control the temperature (λ_{ex} = 360 nm and λ_{em} = 430 nm). After each temperature change or surfactant addition, the samples were allowed to equilibrate for 5 min. The steady-state fluorescence anisotropy was calculated according to Eq. (1):

$$r = \frac{(I_{VV} - I_{VVblank} IG) \times (I_{VH} - I_{VHblank})}{(I_{VV} - I_{VVblank} 2G) \times (I_{VH} - I_{VHblank})} \quad (1)$$

where I_{VV} and I_{VH} are the fluorescence intensities measured parallel and perpendicular to the vertically polarized exciting beam, and G is an intrinsic parameter of the spectrometer ($G = I_{HV}/I_{HH}$) [19,20]. Blank solutions were liposomes of a concentration of 0.1 mM phospholipids.

2.5.2. In vitro release kinetics

In vitro release of liposomal content was monitored using a fluorescence dequenching assay on a Cary Eclipse Fluorescence spectrophotometer [21]. Liposomes hydrated with HEPES buffer containing a chromophore and a collisional quencher, HPTS–DPX (isotonically adjusted), were prepared and filtered to remove the unencapsulated compounds. An aliquot of filtered liposomes was diluted in isotonic HEPES buffer (20 mM HEPES, 144 mM NaCl, pH 7.4, HBS), containing 10 or 50% serum (HBS mixed with 10 or 50% FBS). The HPTS fluorescence of this solution was measured for 1 h at 37 °C. The complete release of encapsulated HPTS/DPX was achieved by the addition of Triton X-100 (final concentration 0.1% *w/w*). The percentage of release at 37 °C was calculated using the following equation:

$$\%release_{37\,^\circ C} = \frac{I_{t,37\,^\circ C} - I_{0,37\,^\circ C}}{I_{T,37\,^\circ C} - I_{0,37\,^\circ C}} \times 100 \quad (2)$$

where $I_{0,37\,^\circ C}$, $I_{t,37\,^\circ C}$, and $I_{T,37\,^\circ C}$ are the fluorescence intensities at time 0, at time t and 1 h after the addition of Triton X-100, respectively.

2.5.3. Transmembrane pH-gradient stability

Experiments to monitor the pH-gradient stability of selected formulations were conducted on a Cary Eclipse fluorescence spectrophotometer. The fluorescence spectra of HPTS as a function of pH (Supplementary Figures S1a) were used to construct a calibration curve of emission intensity ratios as a function of pH (Supplementary Figure S1b) according to a method previously described [22,23]. HPTS (1 μM) in isotonic carbonate buffer (CBS, 25 mM carbonate buffer, 145 mM NaCl, pH 7.8) was adjusted to various pH values (4.5–10.3), and the excitation wavelength λ_{ex} was scanned over the range 330–480 nm. Fluorescence emission was detected at 510 nm. The isosbestic wavelength was 413 nm. The HPTS fluorescence at λ_{ex} = 453 nm and at λ_{ex} = 413 nm of pH-adjusted liposomes in CBS was recorded for 3 h at 25 °C. The complete release of encapsulated HPTS was achieved by the addition of Triton X-100 (final concentration 0.1% *w/w*).

2.6. Interaction between istaroxime and liposomes

The interaction between the free drug and LUVs [24] was studied by DSC. First, a calibration curve was created by mixing increasing drug amount with a fixed volume of LUVs hydrated with CBS (no transmembrane pH gradient applied). Subsequently, a complete drug-loading procedure was performed by dissolving istaroxime in CBS and mixing it with pH-gradient liposomes. Details about the DSC analysis are provided in the Supplementary information.

2.7. Drug loading

DPPC, DOPC, and EPC liposomes in citrate buffer were mixed at different drug-to-lipid ratios (D/L) with 4 mM istaroxime dissolved in CBS. The loading procedure was performed at room temperature and at 50 °C, and the encapsulation efficiency (EE) was measured at t = 10, 30 and 60 min. At the appropriate time points, the unencapsulated drug was removed by centrifuging 300 μL of the mixture on 5000 MWCO Vivaspin500 (Sartorius, Göttingen, Germany) centrifugal filters (15000*g*, 15 min, 20 °C). The filtrate was then recovered and filtered on Chromafil® PTFE filters (Macherey–Nagel GmbH, Düren, Germany) directly in 2-mL screw vials equipped with 200-μL conical glass micro-inserts. The vials were capped with silicone/Teflon-lined screw-caps and placed in the HPLC autosampler (20 °C).

The EE was calculated according to Eq. (3):

$$EE = 100 - \left(\frac{[DB]_{filt}}{[DB]_i} \times 100\right) \quad (3)$$

where $[DB]_i$ and $[DB]_{filt}$ are the initial drug concentration and the drug concentration after the filtration over Vivaspin columns, respectively. Details on the HPLC method are available as Supplementary information.

2.8. Drug release in serum

Ch-BODIPY-marked EPC liposomes were used for drug loading. After 10 min mixing at room temperature, an aliquot of the mixture was diluted 1/20 (*v/v*) in FBS-containing solution (50% *v/v* FBS, 50% *v/v* normal saline), preheated to 37 °C. The unencapsulated drug was not removed prior to dilution in 50% serum. After 10 min incubation at 37 °C under continuous shaking, 2 aliquots of 100 μL were simultaneously loaded on two spin columns in order to perform gel filtration. The spin columns were packed with Bio-Gel® A-15 m (Bio-Rad Laboratories Inc., Hercules, CA) equilibrated with isotonic veronal-buffered saline (VBS, 10 mM sodium barbital, pH 7.4, 145 mM NaCl) and immediately centrifuged (720 *g*, 1 min), optimizing a known method [25]. The liposome content of the fractions was assayed by checking the fluorescence emission intensity of Ch-BODIPY (λ_{exc} = 470 nm, λ_{em} = 520 nm). Those fractions were then recovered from the microplate and analyzed for protein content using an optimized Bradford assay [26]. Fractions collected from the second spin column were subjected to the serum extraction protocol (available as Supplementary information). The reconstituted sample was recovered and analyzed by HPLC, as described in the Supplementary methods.

2.9. Statistical analysis

Statistics were computed with SigmaPlot 11.0 software (SPSS, Chicago, IL). Data, when applicable, are presented as mean ± standard deviation (SD) from at least three separated sample analyzed unless otherwise indicated. The percent release of HPTS/DPX at t = 1 h in HBS and FBS 50% *v/v* and percent encapsulation of istaroxime for each liposome formulation were compared using the one-way analysis of variance (ANOVA), followed by Holm–Sidak's test for *post hoc* comparison in order to ascertain differences between groups. A p value of $\leqslant 0.05$ was considered statistically significant.

3. Results

3.1. Liposome characterization

The effect of PEG–HS content on some physico-chemical features of the liposomes was investigated by means of DLS, DSC, and steady-state fluorescence polarization. Sizes and polydispersity indices (PIs) of each investigated formulation are reported in Fig. 2. No solubilization process took place at the selected PEG–HS concentrations (<15 mol% of the total lipid concentration), as revealed by the almost unaltered hydrodynamic diameters and PIs (0.10–0.15). DSC analysis provided some information on the impact of PEG–HS on the bilayer packing. The extrapolated main transition temperatures (T_m) for the investigated formulations are summarized in Table 1. Complete DSC thermograms are

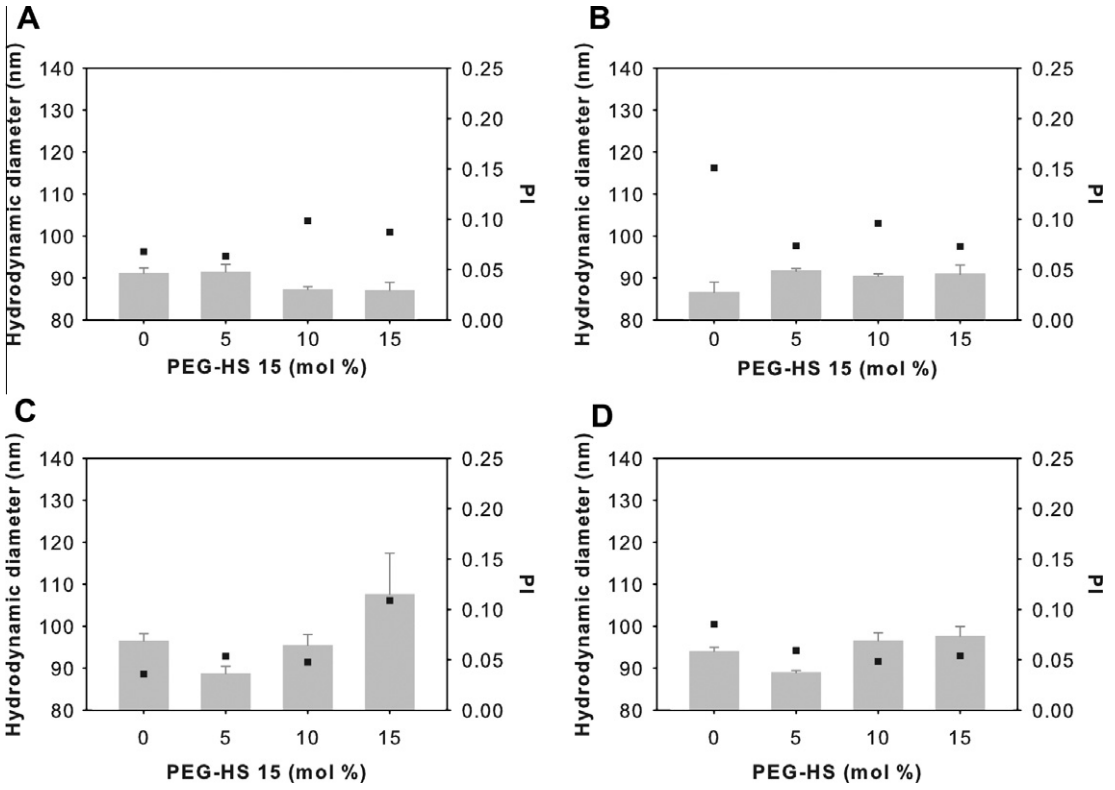

Fig. 2. Mean diameters and PIs of DOPC (A), EPC (B), DMPC (C), and DPPC (D) liposomes as a function of PEG–HS concentration. Mean ± SD (n = 3).

Table 1
Main transition temperatures (T_m) of the investigated liposomal formulations determined by DSC. Mean ± SD (n = 3).

Composition		T_m ± SD (°C)
DMPC		23.3 ± 0.1
	+PEG–HS 5 mol%	23.2 ± 0.1
	10 mol%	23.0 ± 0.1
	15 mol%	22.4 ± 0.1
DPPC		40.8 ± 0.0
	+PEG–HS 5 mol%	40.7 ± 0.1
	10 mol%	40.5 ± 0.1
	15 mol%	40.6 ± 0.1
DOPC		−18.2 ± 0.1
	+PEG–HS 5 mol%	−18.5 ± 0.1
	10 mol%	−18.8 ± 0.0
	15 mol%	−18.8 ± 0.0
EPC		−7.8 ± 0.3
	+PEG–HS 5 mol%	−7.6 ± 0.4
	10 mol%	−7.4 ± 0.3
	15 mol%	Not determined

displayed in Supplementary Figure S2. The thermal analysis confirmed that PEG–HS did not exert a dramatic effect on the bilayer properties. The pre-transition peak, related to the presence of a structured water matrix interacting with the choline group of the phospholipid [27], disappeared in the presence of the surfactant suggesting that it interacted mainly with the polar region of the bilayer [28]. The enthalpy changes observed for increased amounts of PEG–HS in the formulation (Supplementary Figure S3) indicated that the highly cooperative gel to liquid–crystalline phase transition was hampered to some extent [28], and that this effect was more pronounced for saturated lipids. However, the negligible depression of the main T_m suggested that PEG–HS did not induce perturbation in the deep core of the lipid bilayer, localizing preferentially closer to the phosphorous headgroup region of the membrane [29].

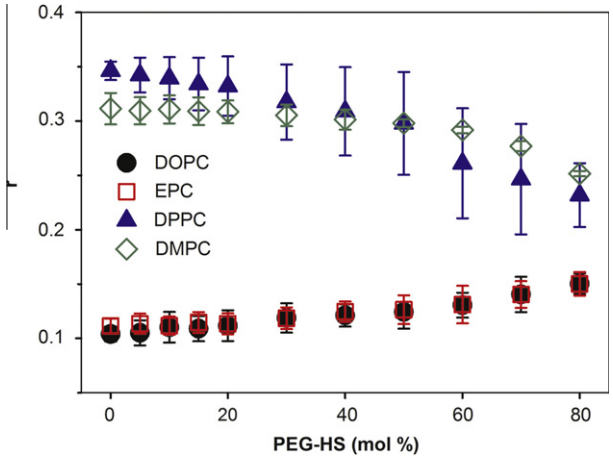

Fig. 3. Variation of steady-state fluorescence anisotropy of DPH in LUVs as a function of PEG–HS concentration: DOPC (●), EPC (□), DPPC (▲), and DMPC (◇) liposomes. The r value for pure PEG–HS micelles is 0.17. Mean ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To obtain further insight into the changes in membrane properties upon addition of PEG–HS, DPH fluorescence polarization in LUVs was measured (Fig. 3) [18]. The anisotropy factor r of the investigated formulations tended to the r value of pure PEG–HS micelles (r = 0.17), showing a solubilization process at very high surfactant concentrations. However, while the fluidity of the DMPC and DPPC bilayer progressively increased, as pointed out by the decrease of the anisotropy factor r, DPH polarization in unsaturated bilayers (EPC and DOPC) increased in the presence of PEG–HS. This unexpected trend could be ascribed to a phase segregation in the liquid–crystalline matrix of the unsaturated PC membranes

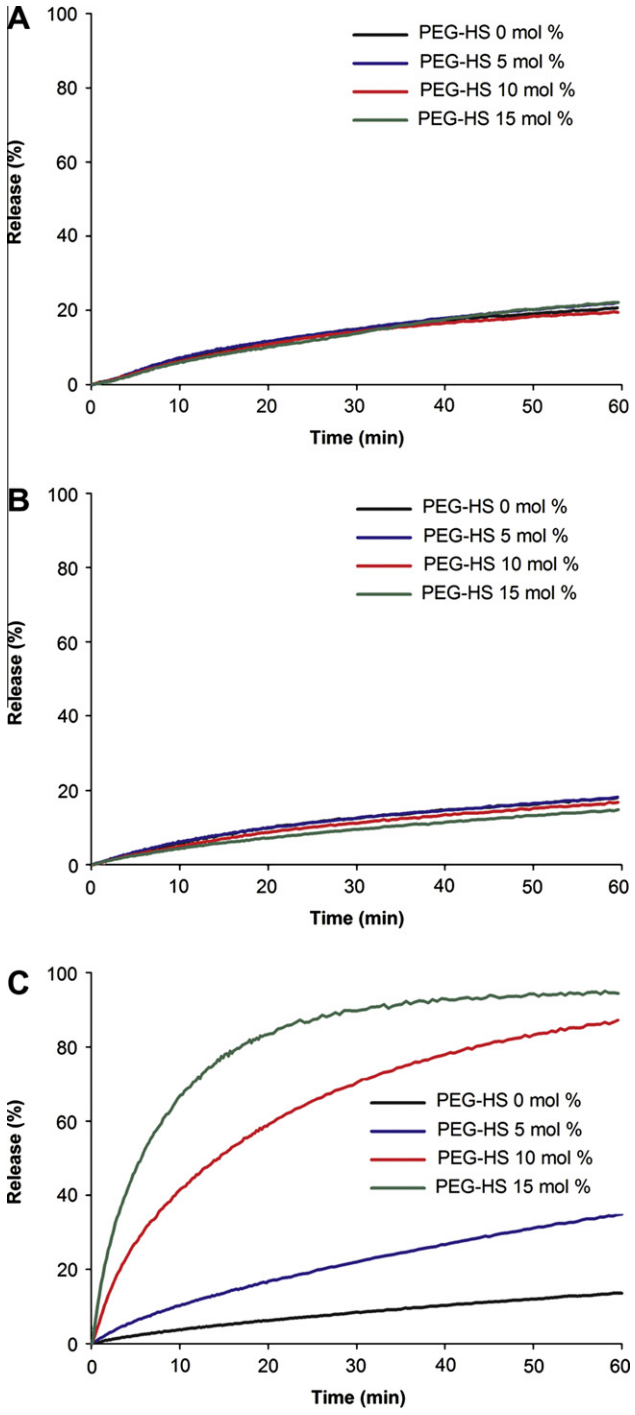

Fig. 4. Release kinetics of DOPC (A), EPC (B), and DPPC (C) liposomes at 37 °C in HBS. Mean ($n = 3$). SD have been omitted for clarity. $p \leqslant 0.05$ for DPPC- *vs* EPC- and DOPC-based formulations after 60 min.

induced by PEG–HS, suggesting a microdomain formation in EPC and DOPC fluid bilayers [30].

3.2. In vitro release kinetics

The release kinetics from liposomes formulated with increasing concentrations of PEG–HS were monitored over 1 h at 37 °C in isotonic buffer, in 10% and 50% serum. Fig. 4 shows the percent of HPTS released as a function of time in HBS. For both EPC and DOPC formulations, the final content release was low in buffer (<25%), and PEG–HS concentration did not affect the bilayer leakiness. On the contrary, PEG–HS strongly affected the release kinetics of DPPC liposomes, inducing a release that increased with PEG–HS content. As shown in Fig. 4C, already after 10 min incubation in buffer, the presence of 10 and 15 mol% PEG–HS produced 43% and 83% content release, respectively. As for DMPC liposomes, the extreme leakiness of DMPC/PEG–HS bilayer (data not shown) let us decide to not further study this formulation.

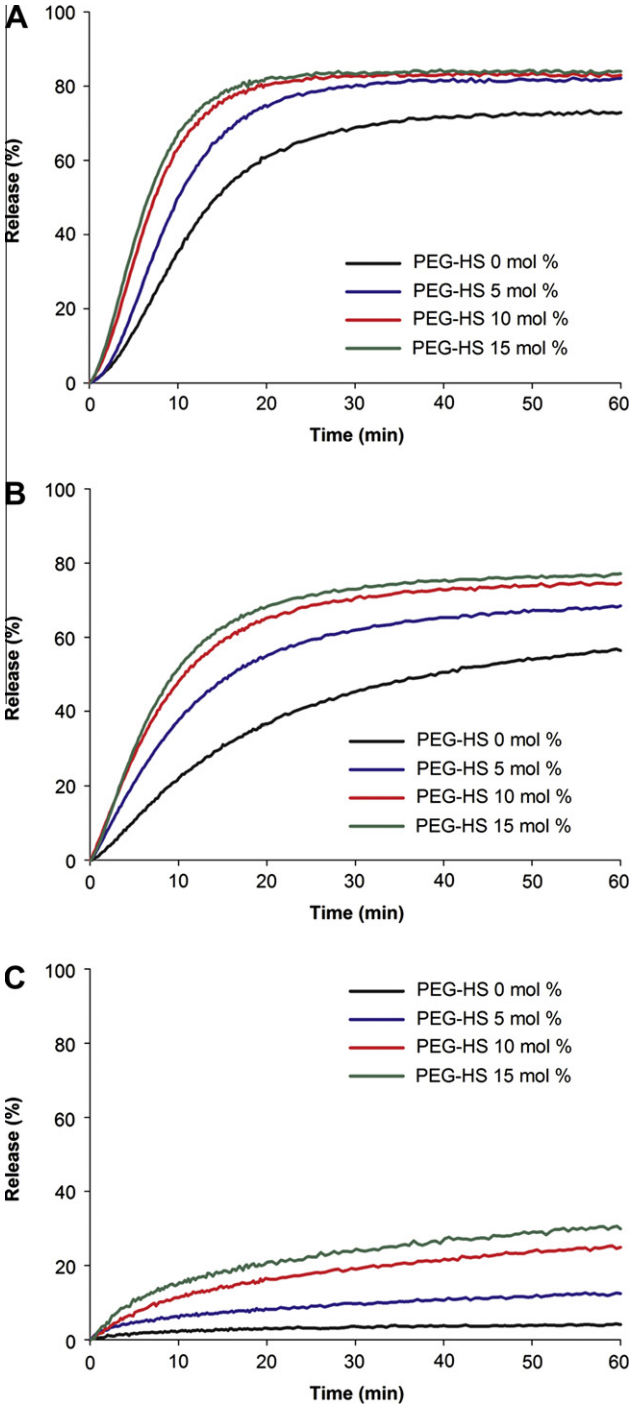

Fig. 5. Release kinetics of DOPC (A), EPC (B), and DPPC (C) liposomes at 37 °C in 50% FBS. Mean ($n = 3$). SD have been omitted for clarity. $p \leqslant 0.05$ for DPPC- *vs* EPC- and DOPC-based formulations after 60 min.

To mimic more physiological conditions, the same experiment was repeated for EPC-, DOPC-, and DPPC-containing liposomes incubated in 10% (Supplementary Figure S4) and 50% FBS (Fig. 5). Serum affected the bilayer stability, inverting the release trend observed upon incubation in buffer. The extent of release from EPC and DOPC formulations (Fig. 5A and B) was higher than 30% already after 10 min incubation. On the other hand, DPPC-based formulations (Fig. 5C) were remarkably stable in serum-containing media, with a final content release ranging from 12% to 30% with increasing PEG–HS concentrations. Notably, in serum (Supplementary Figure S4 and Fig. 5), PEG–HS affected the release kinetics independently of the degree of saturation of the acyl chains in the formulations: the higher the PEG–HS ratio in the bilayer, the greater the release rate.

3.3. pH-Gradient stability

To assess whether a generated transbilayer pH gradient (ΔpH) could be maintained in the PEG–HS liposomal formulations, the fluorescence signal of HPTS encapsulated in liposomes in the presence of ΔpH was measured over time. Typical profiles of HPTS 453/413 nm wavelength ratio (F_R) are shown in Supplementary Figure S5. The calculated F_R value of 0.6 over 3 h for DOPC-based formulations represented a pH ~5.5, still more than two units lower than the external CBS pH. Over the investigated time frame, the DPPC formulations were able to keep a transmembrane ΔpH necessary to perform active drug loading. On the other hand, PEG–HS induced remarkable loss of ΔpH in the EPC formulation at a surfactant concentration of 15 mol% (data not shown).

3.4. Istaroxime/membrane interactions

DSC was chosen to investigate the partition of istaroxime in the lipid bilayer [24]. Due to the amphiphilic nature of the drug, this preliminary study was performed in order to understand the interactions between istaroxime and the phospholipid bilayer. Among the investigated phospholipids (EPC, DOPC, and DPPC), DPPC was chosen for its advantageous thermal behavior, given its narrow melting peak in a temperature range that can be easily analyzed. Thermograms relative to the DPPC bilayer (with and without PEG–HS) incubated with increasing drug molar ratio in the absence of any pH gradient (in CBS, pH 7.8) are shown in Fig. 6. The extent of the interaction occurring between DPPC and istaroxime was evaluated by analyzing the resulting thermograms. Istaroxime induced a progressive depression of DPPC main T_m in a concentration-dependent manner, both without and with PEG–HS (Fig. 6A and B, respectively). In the thermograms, a unique main peak was identified, meaning that istaroxime uniformly localized in the bilayer. The linear decrease of the main T_m as a function of the drug molar fraction (Supplementary Figure S6) suggested a strong interaction of the drug with the acyl chains [29].

To evaluate the effect of istaroxime on DPPC bilayer in the presence of a ΔpH, the drug was incubated with pH-gradient liposomes. The incubation was performed at room temperature and above DPPC T_m (50 °C). Aliquots of the mixtures were withdrawn at different times (t = 0, 30 and 60 min) and analyzed. Fig. 7A and B shows the thermograms for the DPPC formulations incubated at room temperature and at 50 °C, respectively. When the liposome/drug mixture was incubated at room temperature, a phase separation was observed at all times, indicating a non-uniform distribution of istaroxime in the DPPC bilayer. Thermograms obtained after incubation at 50 °C showed a less-defined phase separation represented by a gradual broadening over time of the main peak, presumably originating from the merging of the original main peak with the small shoulder. By comparing Figs. 6 and 7, it seems evident that in the presence of ΔpH, the interaction between the drug and the lipid bilayer changed. The linear T_m depression observed when istaroxime was mainly in its neutral form can be explained by a possible homogeneous distribution of the drug within the bilayer. The phase separation represented by the presence of a shoulder in the thermogram suggests that the protonated form of the drug induced an anisotropic distribution of the amphiphilic components in the bilayer (Fig. 7). Thermal treatment seemed to play a role as well. Incubation above T_m of DPPC might have induced a loss of pH gradient, thereby increasing the amount of istaroxime in the neutral form (Fig. 7B). Thermograms for DPPC/PEG–HS formulations are reported in Supplementary Figure S7. When PEG–HS was added in the formulation, no phase separation was observed. Immediately after drug addition, the thermal shift was noticeable (Supplementary Figure S7, blue curve). It is likely due to a stronger interaction of the drug with the bilayer in the first moments after the pH gradient was established. After 30 and 60 min, the T_m shifted back almost to the original value, pointing out that almost no drug was associated to the lipid bilayer. This may suggest a role of PEG–HS in reducing the interaction of the drug with the bilayer once ΔpH is generated (compare Fig. 6B and S7).

3.5. Drug loading

EPC, DOPC, and DPPC were chosen as the main components in the investigated formulations for drug loading. Pure lipid formulations were compared to the PEG–HS-based ones (5 and 10 mol%). The D/L (D/L = 0.2, 0.3, and 0.45) and temperature were varied. Preliminary tests conducted at D/L = 0.6 and 1.5 (data not shown) indicated that the drug-loading efficiency was generally below 50% for D/L = 0.6 and below 30% for D/L = 1.5. While DOPC-based formulations were not affected by incubation at 50 °C (% loading remained almost

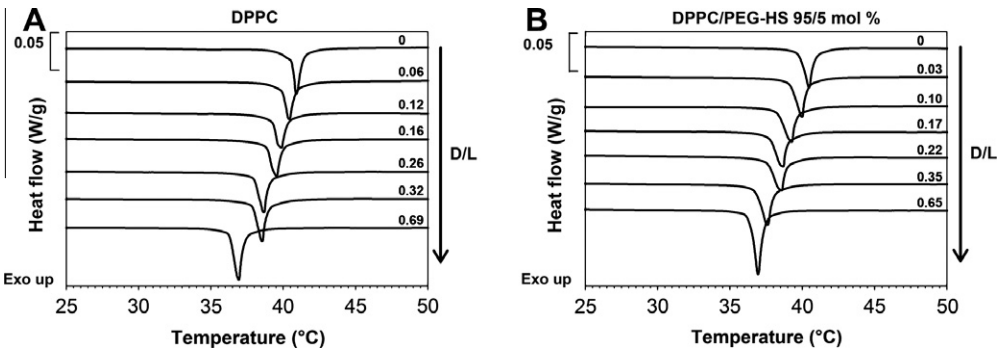

Fig. 6. DSC thermograms of pure DPPC (A) and DPPC/PEG–HS 95:5 mol% (B) vesicles incubated with increasing D/L in the absence of ΔpH. Thermograms have been offset for clarity.

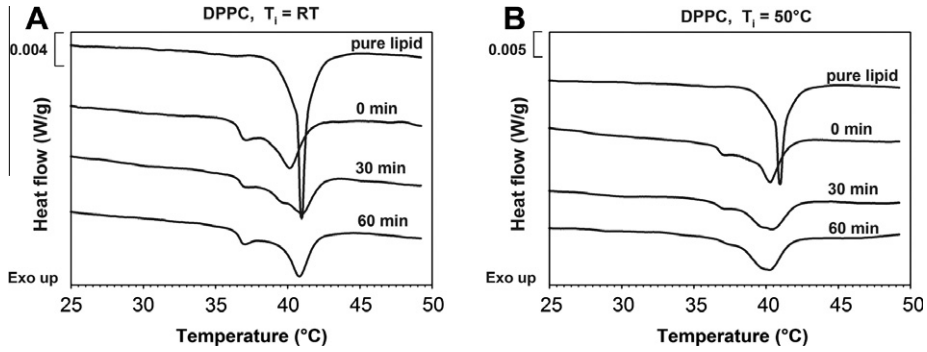

Fig. 7. DSC thermograms of istaroxime loaded in pure DPPC vesicles (D/L = 0.3) after incubation at room temperature (A) and at 50 °C (B) in the presence of ΔpH at different time points (t = 0, 30 and 60 min).

Table 2
Istaroxime loading in the selected liposomal formulations after 10 and 60 min incubation. Mean ± SD (n = 3). The p values are reported in Tables S2 and S3.

Composition	D/L ratio	EE after 10 min (%)	EE after 60 min (%)
DOPC	0.20	71.3 ± 2.1	72.9 ± 1.8
	0.30	71.9 ± 2.5	74.5 ± 2.4
	0.45	64.1 ± 1.5	66.3 ± 1.6
EPC	0.20	75.0 ± 1.4	74.8 ± 2.7
	0.30	73.7 ± 4.3	77.9 ± 2.7
	0.45	64.7 ± 1.2	67.2 ± 2.1
EPC/PEG–HS (95/5 mol%)	0.20	73.5 ± 0.9	69.7 ± 1.4
	0.30	70.7 ± 4.6	67.1 ± 5.6
	0.45	55.2 ± 7.7	53.0 ± 1.0
EPC/PEG–HS (90/10 mol%)	0.20	66.7 ± 1.1	58.4 ± 2.2
	0.30	59.5 ± 3.0	50.4 ± 5.1
	0.45	53.0 ± 1.2	43.7 ± 2.2

unaltered), DPPC-based liposomes encapsulated remarkably less drug (data not shown). Incubation above DPPC T_m likely induced a pH-gradient loss through the bilayer, implying lower efficiency in trapping the protonated istaroxime inside the liposomal core. The EE for pure EPC, pure DOPC, and for EPC formulations containing 5 and 10 mol% PEG–HS is reported in Table 2. The high lipid concentration needed for the drug loading (166 mM) did not allow to investigate DOPC/PEG–HS and DPPC/PEG–HS liposomes further, since PEG–HS induced gelation of the dispersions at all tested concentrations ($\geqslant$5 mol%).

An incubation time of 10 min was sufficient to achieve the highest encapsulation efficiency in almost all the formulations. Loading efficiency in the formulation containing 10 mol% of PEG–HS was the lowest observed (<67%). This amount of surfactant might have favored a proton efflux already after 10 min, leading to a presumable pH-gradient loss. This helped in placing 5 mol% as the highest limit in PEG–HS content in the drug release studies. The high and stable drug encapsulation efficiency achieved in EPC liposomes, obtained with a fast incubation at room temperature, resulted to be optimal for formulating istaroxime. The release in serum was thus investigated for EPC and EPC/PEG–HS 95/5 mol formulations (*vide infra*).

3.6. Istaroxime release in serum

Initially, pure istaroxime dissolved in CBS was incubated 10 min in 50% serum at 37 °C and then filtered over Bio-Gel spin columns. Drug extraction from the eluted serum-containing fractions was performed, and the reconstituted extracted organic phases were analyzed by means of HPLC (data not shown). By comparing the elution profile of pure istaroxime and of pure Ch-BODIPY-marked liposomes, almost no overlap was observed (Supplementary Figure S8). Istaroxime release was monitored by incubating Ch-BODIPY-marked EPC formulations (pure lipid and 5 mol% PEG–HS) in 50% serum at 37 °C. Typical liposome/drug elution profiles are reported in Fig. 8. Istaroxime release from the selected liposomal formulations was almost complete after only 10 min incubation in 50% serum, as clearly shown by the non-overlapping elution profiles. By comparing the area below the curve of the eluted pure drug incubated in serum with the eluted drug from EPC liposomes (with and without PEG–HS), the percentages of released drug were almost 100% (data not shown).

4. Discussion

Whenever a drug is responsible for pain upon injection, suspensions or emulsions are preferred to a normal aqueous solution [13]. Alternatively, the drug can be encapsulated in a biocompatible/bio-

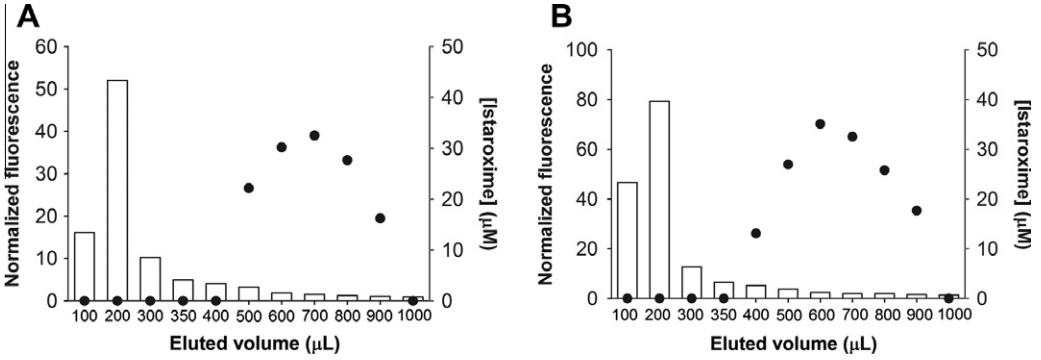

Fig. 8. Typical elution profiles of pure EPC (A) and EPC/PEG–HS 95:5 mol% (B) vesicles, loaded with istaroxime and incubated 10 min in 50% FBS. Bars: Ch-BODIPY-marked liposomes; circles: istaroxime.

degradable carrier such as mixed micelles or liposomes. Among the several drug delivery nanocarriers [31], liposomes are a versatile tool for achieving efficient drug encapsulation and triggered release upon specific stimuli [32]. Introducing appropriate variations of the bilayer formulations, the physico-chemical properties of the liposomes can be modulated, and specific behavior in biological media can be established or amplified.

In the present study, vesicles, formulated with phospholipids and PEG–HS, an excipient chosen to modulate the bilayer properties, were selected as drug delivery system for istaroxime. Istaroxime is a relatively stable compound in its dry form. In solution, it epimerizes at basic pH values, while the oxime gets hydrolized under acidic conditions (Supplementary Figure S9). Therefore, in order to preserve its chemical integrity, istaroxime should be stored preferentially in the dry form. Reconstitution could thus take place just prior to administration by means of an active *in situ* loading in liposomes, as previously described for the Myocet® formulation [16].

Several liposomal formulations were screened in order to identify one which would allow a fast and efficient drug loading while releasing the drug rapidly once in the serum. Liposomes prepared with DPPC were too stable in serum. Fast release in serum was sought to avoid substantial changes in the pharmacokinetic parameters; therefore, DPPC-based formulations were not further investigated. Even the addition of PEG–HS up to 15 mol%, introduced to accelerate the release rate, led to insufficient leakage in serum (Fig. 5). This result was unexpected given the strong influence on release rate that PEG–HS had in buffer (Fig. 4).

In buffer, release from liposomes is reported to be faster at temperatures approaching the gel to liquid–crystalline phase transition [33,34]. In proximity of the transition temperature, mismatch formation in the interfacial liquid–solid boundaries leads to an increased permeability of the lipid membrane [33,35]. For pure DPPC bilayer (T_m = 41 °C), no leakage is expected for temperature lower than 39 °C [33,34]. When PEG–HS was added to the formulations, a release linearly dependent on the surfactant was observed already at 37 °C. This observed higher leakage suggests the presence of defects in the interfacial solid–liquid boundaries, possibly induced by the interaction of the 12-hydroxystearic chain of PEG–HS with the bilayer. Serum-induced destabilization of liposomes is supposed to be due to the association of apolipoproteins with the bilayer. It occurs more easily when the latter is in its liquid–crystalline phase or presents phase boundaries [36]. In case of DPPC liposomes, the tight packing of saturated acyl chains in their gel-phase made less probable the insertion of serum protein in the lipid bilayer [36,37].

EPC- and DOPC-based formulations were stable in buffer. At none of the investigated concentrations PEG–HS affected the permeability of the bilayers, already in their liquid–crystalline phase. On the other hand, the unsaturated formulations were leaky in serum, allowing fast release. The insertion of apolipoproteins is favored in unsaturated membranes given their fluidity at physiological temperature. This fluidity promotes phospholipid removal, pore formation in the bilayers, and increased permeability [37]. The addition of PEG–HS, by inducing the formation of defects in the unsaturated bilayer, exerted a key role in modulating the release in serum in a concentration-dependent manner. PEG–HS did not seem to interact deeply with the inner hydrophobic core of the bilayer, since the melting profiles were preserved, and the T_m was almost unaltered. As suggested by the suppression of the pre-transition peak, the lipophilic part of the surfactant, *i.e.* the mono- and di-esters of 12-hydroxystearic acid, interacted mainly with the bilayer region close to the polar headgroup of the phospholipids. The extent of perturbation of the bilayer cooperativity, induced by the addition of PEG–HS, turned out to be an advantage in terms of serum-triggered destabilization.

The good stability in preserving a transmembrane pH gradient over time shown by low T_m liposomes (EPC and DOPC) in buffer was beneficial with respect to the loading process. It allowed reaching a rapid and high istaroxime encapsulation at room temperature. An increase in the incubation temperature did not improve the encapsulation efficiencies. DPPC-based formulations lost their ΔpH above T_m, resulting in poor istaroxime uptake, whereas the unsaturated phospholipids, already in the liquid–crystalline phase, were fluid enough to ensure the highest uptake possible at the selected conditions. As pointed out by thermal analyses, in the presence of a transmembrane pH gradient, the drug interacted with the bilayer. When protonated, istaroxime induced a phase separation in the membrane, while in its neutral form, it seemed to homogeneously intercalate in the lipid bilayer, as the linear dependency of T_m shift on istaroxime concentration suggested. The most efficient formulations in terms of drug loading were also the most easily destabilized in serum, pointing out liquid–crystalline phase formulations as the best liposomal dosage forms for istaroxime. EPC was better than DOPC because, at high lipid concentration, PEG–HS induced gelation of the DOPC suspension when present at $\geqslant$ 5 mol%. For EPC and DOPC liposomes, the addition of PEG–HS at more than 5 mol% was found to negatively impact the encapsulation efficiency. Moreover, PEG–HS lowered the interaction of istaroxime with the bilayer once the transmembrane pH gradient was generated. Based on the PC thermograms, we hypothesized that PEG–HS intercalated in the outer part of the bilayer, interacting with the region close to the polar headgroups. This may have in return impeded the interaction of the drug with the membrane through steric hindrance.

When formulated in EPC-based liposomes, istaroxime was rapidly released in serum (<10 min), suggesting that the observed interactions of the drug with the bilayer represented a small fraction of the drug or were not strong enough to interfere with the release. Unfortunately, due to this fast release kinetics and lack of sensitivity of the assay within this time range, the PEG–HS effect could not be measured. The *in vitro* release kinetics conducted with HPTS suggest that PEG–HS could be further added to accelerate istaroxime release, should subsequent clinical studies reveal that the pharmacokinetics of the drug is altered by its incorporation into the liposomes.

5. Conclusions

Vesicles with various compositions were prepared, and the effect of a selected excipient on the physico-chemical features of the lipid bilayer was evaluated by means of different techniques. Release kinetics of some formulations were monitored in physiological conditions in order to determine the ability of PEG–HS to modulate membrane permeability. Low T_m liposomes were identified as the best formulations in terms of release rates in serum-containing media. The impact of temperature and drug-to-lipid ratios on efficient istaroxime active encapsulation was assessed. EPC formulations enabled encapsulation efficiency higher than 70% at room temperature and almost complete release of istaroxime upon incubation in physiological conditions.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2011.04.013.

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