Research paper

Stealth nanocarriers based sterosomes using PEG post-insertion process

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1. Introduction

The development of nanotechnology has already provided medical and pharmaceutical benefits [1]. Liposomal nanocarriers is one example of a real-life nanomedicine that has already been commercialized for various therapies: Visudyne (Novartis Europharm Ltd) against age-related macular degeneration (drug: Verteporfin); Ambisome® (Gilead Sciences) against fungal infection (drug: Amphotericin B). In the field of cancer research, liposomal formulations of chemotherapy drugs are already on the market. For examples Caelyx® (Janssen Cilag Internat Nv) and Myocet® (Cephalon Europe) are used to treat Kaposi’s sarcoma, ovarian and metastatic breast cancer (drug: Doxorubicin); and Daunoxome® (Galene Limited) for Kaposi’s sarcoma therapy (drug: Daunorubicin).

Clinical results have been a springboard for liposome research which is growing continuously in order to improve liposome efficacy [2–4]. In order to be used intravenously, the surface of liposomes must be modified to obtain long-term circulation in the blood stream, by decreasing capture by the monocytic phagocyte system (MPS) [5]. Polyethylene glycol (PEG) is the most common polymer used for this purpose [6]. Nevertheless, as with other nanocarriers, liposomes can present some drawbacks, such as low encapsulation rates, early release of hydrophilic drugs, and moderate stability, depending on the encapsulated drugs [7].

Sterosomes (STEs), a new promising class of liposomes, are formed without phospholipid and include a high-cholesterol content. They are produced from a binary mixture of cholesterol (Chol) and a single chain amphiphile, such as palmitic acid (PA), of moderate stability, depending on the encapsulated drugs [7].

Sterosomes (STEs), a new promising class of liposomes, are formed without phospholipid and include a high-cholesterol content. They are produced from a binary mixture of cholesterol (Chol) and a single chain amphiphile, such as palmitic acid (PA). This composition confers particularly interesting properties, including high chemical stability and low bilayer permeability [8]. Chol and PA do not form fluid bilayers individually, but once mixed together under particular conditions (pH > 7, Chol molar fraction of 0.7), a fluid bilayer, in the liquid-ordered phase, is obtained [9]. These fluid bilayers can be extruded using classical extrusion processes to form large unilamellar vesicles whose diameter is controlled by the filter pore size of the extrusion system and displays a narrow, and monomodal distribution. STEs show an...
improved impermeability (with calcine and $^{14}$C-glucose) in comparison to traditional phospholipid liposomes, due to a considerably higher Chol content than the saturation limit generally reported for phospholipid bilayers [8]. STEs require a surface modification to ensure stealth properties after systemic administration. Cui et al. reported the incorporation of PEG-modified cholesterol (PEG-Chol) into the initial mixture of PA-Chol, before lipid hydration and the extrusion process [10]. Up to 20% of PEG-Chol (molar ratio) was introduced in the mixture without significant structural change of the STEs. PEG-modified STEs maintained very limited permeability to calcine and doxorubicin. Moreover, pharmacokinetic studies on murine models exhibited a decrease in the clearance of PEG-modified STEs compared to the non-modified ones.

The inclusion of PEGylated amphiphiles in initial mixtures prior to hydration leads to the presence of PEG on both leaflets of liposomes, a phenomenon that led to drug trapping modifications and rapid liposome destabilization in some cases [11–13]. Alternatively, in phospholipidic liposomes, PEG can also be introduced at the liposome surface by the addition of PEGylated amphiphiles once the liposomes are obtained [11,13–15]. In this work, the applicability of this strategy was investigated. It was not obvious that PEGylated amphiphiles could insert in significant amount post-insertion of DSPE-PEG with various PEG chain lengths was done with non-modified STEs for comparison. The stability of PEG-modified and non-modified STEs was evaluated (i) upon storage at 4 and 37°C for 7 days, (ii) as a function of pH (at room temperature). Stability of Dil and DiD-loaded, non-modified and PEG-modified STEs was also evaluated in serum, mixing equal volumes of STE suspensions and 10% heat-inactivated fetal bovine serum (FBS) (Lonzafarma, Verviers, Belgium), at 37°C for 48 h under gentle oscillation.

2.3. Sterosome-surface modification with PEG chains

DSPE-PEG in PBS (500 µL) was added to 500 µL of a STE suspension (7 mg mL$^{-1}$). The final concentrations of DSPE-PEG were 0, 0.5, 1, 2.5, or 5 mg mL$^{-1}$, leading to a DSPE-PEG/PA-Chol incubation ratio of about 0, 7, 14, 36, and 72% (w/w), respectively. Mixtures were gently stirred for 7 h at 37°C or 50°C to determine the surface-modification kinetics. Except for the kinetics studies, the process to obtain PEG-modified STEs was carried out at 37°C for 3 h. PEG-modified STE isolation was performed using a dialysis device (membrane cut-off: 15 kDa) against PBS for 2 days, changing the buffer three times a day. This isolation protocol was also done with non-modified STEs for comparison. The stability of PEG-modified and non-modified STEs was evaluated (i) upon storage at 4 and 37°C (pH = 7.4) and (ii) as a function of pH (at room temperature). Stability of Dil and DiD-loaded, non-modified and PEG-modified STEs was also evaluated in serum, mixing equal volumes of STE suspensions and 10% heat-inactivated fetal bovine serum (FBS) (Lonzafarma, Verviers, Belgium), at 37°C for 48 h under gentle oscillation.

2.4. Sterosome characterization

The Z-average diameter (Z-Ave), the polydispersity index (Pdi), and the zeta potential (ZP) of PEG-modified and non-modified STEs were determined during the surface-modification, purification process and stability evaluation, by dynamic light scattering using a Zetasizer® Nano ZS (Malvern Instruments S.A., Worcestershire, UK). The scattered light from a helium–neon laser, 4 mW, 633 nm, was recorded at an angle of 173° whereas the temperature was maintained at 25°C. The curve fitting of the correlation functions was performed using an exponential fit (Cumulant approach) for Z-Ave and Pdi determination. The Smoluchowski approximation was used to determine the electrophoretic mobility required for ZP determination. All STE suspensions were diluted (60-fold dilution factor) in PBS before performing measurements in triplicate.

Fluorescence emission intensity of dye-loaded STE suspensions was measured during the stability experiment (in PBS medium), using a microscope reader Fluoroscan Ascent® (Labsystems SA, Cergy-Pontoise, France). Filter pairs with adequate excitation and emission wavelength coupling ($\lambda_{ex}$–$\lambda_{em}$) were used: 544–590 and 646–678 nm for Dil and DiD, respectively.

2.5. CH50 experiment

Complement consumption was tested on normal human serum (NHS) by measuring the residual hemolytic capacity of the complement after incubation with various STEs. The assay was based on the CH50 experiment described elsewhere [17]; CH50 corresponds to the concentration of 0.5 mg g$^{-1}$ of PA-Chol. The freeze-dried lipid mixtures (with or without dyes) were hydrated with saline phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$ and 1.76 mM KH$_2$PO$_4$, pH = 7.4) (PBS). The suspensions were subjected to 5 cycles of freezing-and-thawing (from liquid nitrogen temperature to 75°C) and vortexed between successive cycles to ensure a good hydration of the samples. The pH of the suspension was measured and readjusted to pH 7.4 if necessary by the addition of a diluted NaOH solution. The final lipid concentration was 7 mg mL$^{-1}$. STEs were obtained by extrusion using a handheld Liposofast extruder (Avestin, Ottawa, Canada). The dispersions were extruded through two, stacked polycarbonate filters (100-nm pore size) 15 times, at about 75°C. For each STE preparation, the experiments were repeated 3 times.

2. Materials and methods

2.1. Materials

Cholesterol (Chol) and palmitic acid (PA) were supplied by Sigma Chemical Co. (Saint-Quentin-Fallavier, France). 1,1′-dioctadecyl-3,3,3′,3′-tetramethyldilcarboxyanine perchlorate (DiI) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethyldilcarboxyanine 4-chlorobenzenesulfonate (DiD) were supplied by Life Technologies SAS (Saint-Aubin, France). Sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na$_2$HPO$_4$) and potassium phosphate monobasic (KH$_2$PO$_4$) were purchased from Prolabo (Fontenay-sous-Bois, France). Deionized water was obtained from a Milli-Q plus® system (Millipore, Billerica, MA, USA). Methanol and benzene were supplied by Fisher Scientific (Illkirch, France). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)] (DSPE-PEG) with various polyethylene glycol (PEG) chain lengths (1, 2, 3, and 5 kDa) was provided by Avanti Polar Lipids Inc. (Alabaster, AL, USA). All solvents and materials were used as received.

2.2. Non-loaded and loaded sterosome preparation

Weighted amounts of PA and Chol were mixed and dissolved in a benzene/methanol (95/5 v/v) mixture. The PA/Chol molar fraction was fixed at 0.3/0.7. The solution was frozen in liquid nitrogen and lyophilized for at least 16 h to allow the complete sublimation of the organic solvents. For Dil and DiD loadings, the fluorescent dyes were added into the initial mixture before solvent sublimation at 37°C.
to the amount of NHS (Etablissement Français du Sang, Angers, France) able to lyse 50% of a fixed number of sheep erythrocytes sensitized with rabbit (anti-sheep) erythrocyte antibodies (EuroBio, Courtaboeuf, France).

Briefly, STE suspensions at various concentrations were added to NHS (1 mL final volume and 4-fold dilution factor for NHS). After a 1-h incubation at 37°C under gentle stirring, the suspensions were diluted (1/25 v/v) in VBS2 (Veronal buffered saline containing 0.15 mM Ca2+ and 0.5 mM of Mg2+). Diluted STE suspensions were added to sensitized sheep erythrocytes (10⁶ cells mL⁻¹). The reaction was stopped by adding ice-cold NaCl solution (0.15 M) after 45-min incubation at 37°C under gentle stirring. After centrifugation (10 min, 800g), non-lysed erythrocytes formed a pellet. The optical density of the supernatant was measured at 415 nm (wavelength of the hemoglobin absorption maximum) using a microplate reader (Multiskan Ascent, Labsystems SA, Cergy-Pontoise, France), and compared to control NHS without STEs. Control experiments evaluated the spontaneous erythrocyte hemolysis due to STEs alone. All the experiments were repeated in triplicate.

2.6. Macrophage uptake

THP-1 cells (human monocyte/macrophage cell line obtained from ATCC, Manassas, VA, USA) were grown in suspension at 37°C, 90% humidity, 5% CO₂ in an ATCC medium. Cell differentiation was induced in the same medium by adding phorbol 12-myristate 13-acetate (200 mM) (PMA, Sigma, Saint-Quentin-Loire, France). A 24-h incubation allows the cells to adhere. After medium aspiration to eliminate non-adhered cells, fresh medium was added and the cells were incubated under the same conditions for 48 h. The cells (5·10⁴ cells/mL⁻¹) were then plated on a 24-well cell culture dish and left to grow for 24 h at 37°C. Dil-loaded, non-modified or PEG-modified STE suspensions (3.85 mg mL⁻¹) were deposited on the cell monolayer and incubated for 90 min at either 4°C or 37°C. The amount of phagocytized STEs was determined quantitatively in a FACScan flow cytometer with the CellQuest Software (BD Biosciences, Le Pont-de-Clax, France). All experiments were repeated in triplicate.

2.7. In vivo experiments

Female nude SCID mice (18–22 g) (Harlan, Gannat, France) were housed and maintained at the University animal facility (SCAHU). All the animal experiments were performed in agreement with the EEC guidelines and the “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985) and with the agreement of Comité d’Éthique pour l’Expérimentation Animale des Pays-de-la-Loire (authorization CEEA; 2012-79). The mice (n = 3 for each group) were anesthetized with 112.5 mg ketamine per kg body weight and 1.5 mg xylazine per kg body weight. 100 µL of non-modified or PEG-modified STEs containing DiD (dose of 32.5 µg kg⁻¹), were intravenously administered (caudal vein). Fluorescence imaging and analysis of the whole mouse body was then carried out, and subsequently the ex vivo organs were analyzed using an in vivo fluorescence imaging system (Cambridge Research Instruments Maestro™, Woburn, MA, USA). Pictures were obtained by using a time exposition of 500 ms between 630 and 800 nm.

2.8. Statistical analysis

Significant differences between samples were analyzed by the Kruskal-Wallis test, followed by Nemenyi-Dunn’s post hoc test for pairwise comparisons.

3. Results

3.1. Non-modified and PEG-modified sterosome characterization

STEs are produced after the extrusion of a binary mixture of palmitic acid (PA) and cholesterol (Chol), with a 0.3/0.7 molar fraction [8]. They have an unilamellar spherical structure, whose diameter is determined by the 100-nm pore diameter of the filters used for the extrusion (Table 1). PdI values lower than 0.2 indicated a monomodal and monodispersed distribution. The high negative ZP value for non-modified STEs (about −40 mV) was due to the negative charge carried by unprotonated PA (palmitate ion) at pH = 7.4 [8]. Lipophilic dyes (DiI and DiD) loaded into the STE bilayer did not affect the size and ZP values (Table 1).

The presence of interfacial PEG introduced by the post-insertion process could be probed by ZP measurements (Fig. 1 and Table 1). Fig. 1 presents Z-Ave and ZP variations of STEs during the process of DSPE-PEG post-insertion at 37°C. The effects of PEG chain length, from 1 to 5 kDa, and the effect of the ratio DSPE-PEG/PA-Chol, from 7 to 72% (w/w) were characterized. The main feature associated with the presence of interfacial PEG is an increase of ZP values during incubation, reaching values close to neutrality (lower than −5 mV). The kinetics of the ZP increase was faster for longer PEG chain, and for higher the DSPE-PEG/PA-Chol ratio. As an illustration, it took about 300 min to reach a ZP of about −20 mV for STEs incubated with DSPE-PEG 1 kDa at a ratio DSPE-PEG/PA-Chol of 7% (w/w), whereas this ZP value was reached after only 90 min if the ratio was 36% (w/w), or if the PEG chain length was increased to 3 kDa. The STE sizes were not significantly affected throughout the post-insertion process (Fig. 1). The PdI values remained lower than 0.2, showing that PEG-modified STE size distribution remained monomodal and monodispersed (data not shown).

A similar surface modification was performed at 50°C (see supplementary information, Fig. S1). For all the investigated experimental conditions, the profiles of ZP variation showed a rapid increase of ZP values as a function of incubation time, similar to those obtained at 37°C. The increase of temperature accelerated DSPE-PEG insertion in STEs as inferred from the ZP changes. Similar to the incubation at 37°C, STE size distribution was not affected by DSPE-PEG insertion (see supplementary information, Fig. S1).

As an incubation of 3 h at 37°C provided quasi electroneutrality for all the investigated PEG chain length, these parameters were chosen for the PEGylation process of STEs. Modified and non-modified STEs were dialyzed for the following experiments. The STE concentrations were evaluated using derived count rate tool from dynamic light scattering measurements, as previously described [18]. No significant change of STE concentration was observed upon dialysis. PEG-modified STEs characteristics are described [18]. No significant change of STE concentration was observed throughout the post-insertion process (Fig. S2) for 5 months. Non-modified STEs remained stable (constant Z-Ave and ZP values), as already reported in the literature [8]. PdI values remained lower than 0.2 (data not shown). The surface modification did not affect stability. For a DSPE-PEG/PA-Chol ratio of 7 or 72% (w/w), and PEG chain lengths of 2 or 5 kDa, the size evolution remained similar to non-modified STEs, i.e. constant Z-Ave, PdI, and ZP values. Moreover, no change in fluorescence emission intensity and size distribution for DiI and DiD-loaded, PEG-modified and non-modified STE suspension, confirmed the
stability of STEs (shape and fluorescence labelling) in presence of FBS up to 48 h (data not shown).

Non-modified PA/Chol STEs were destabilized when pH decreased (Fig. 3), as already shown in the literature [8]; this response was associated with the protonation of PA that leads to the phase separation of the STE components. At pH values higher than 6, Z-Ave and PdI values remained stable at about 120 nm and 0.13, respectively. At pH 5.5, the Z-Ave value increased to 450 nm and a large and polydispersed distribution of particles was obtained for lower pH values. Conversely, PEG-modified STEs were found stable over the whole investigated pH range; for DSPE-PEG/PA-Chol ratio at 7 or 72% (w/w), and a PEG chain lengths of 2 or 5 kDa, the size and polydispersity values were the same as those obtained after extrusion at pH = 7.4, i.e. about 130 nm and 0.13, respectively.

3.3. Sterosome stealth capacity in relation to the complement system

The consumption of CH50 units was measured at a fixed amount of human serum in the presence of increasing STE concentrations. Its increase was indicative of complement activation by STEs (Fig. 4). On one hand, non-modified STEs led to rapid CH50 unit consumption, with a total consumption at a non-modified STE concentration of 50 µg/mL. On the other hand, the consumed CH50 unit increased only to about 10% for PEG-modified STEs at the same concentration. The PEG-modified STE concentration had

Table 1
PEG-modified and non-modified, loaded and non-loaded sterosome (STE) characterization: Hydrodynamic diameter (Z-Ave), polydispersity index (PdI) and zeta potential (ZP), used for complement activation, macrophage uptake and in vivo studies. STEs were modified using DSPE-PEG, whose PEG chain lengths were 2 or 5 kDa. R is the ratio DSPE-PEG/PA-Chol (w/w). (n = 3, mean ± SD).

<table>
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<tr>
<th>Loading</th>
<th>Z-Ave (nm)</th>
<th>PdI</th>
<th>ZP (mV)</th>
</tr>
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<tbody>
<tr>
<td>Complement activation study</td>
<td>Non-modified STEs</td>
<td>120 ± 5</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>STEs, PEG 2 kDa, R = 7%</td>
<td>131 ± 2</td>
<td>0.12 ± 0.03</td>
<td>−10 ± 3</td>
</tr>
<tr>
<td>STEs, PEG 2 kDa, R = 72%</td>
<td>122 ± 10</td>
<td>0.10 ± 0.01</td>
<td>−8 ± 2</td>
</tr>
<tr>
<td>STEs, PEG 5 kDa, R = 72%</td>
<td>125 ± 2</td>
<td>0.18 ± 0.01</td>
<td>−6 ± 1</td>
</tr>
<tr>
<td>Non-modified STEs</td>
<td>128 ± 2</td>
<td>0.09 ± 0.02</td>
<td>−4 ± 2</td>
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<tr>
<th>Loading</th>
<th>Z-Ave (nm)</th>
<th>PdI</th>
<th>ZP (mV)</th>
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<tbody>
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<td>136 ± 2</td>
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<td>STEs, PEG 2 kDa, R = 7%</td>
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<td>0.06 ± 0.01</td>
<td>−10 ± 2</td>
</tr>
<tr>
<td>STEs, PEG 2 kDa, R = 72%</td>
<td>138 ± 1</td>
<td>0.07 ± 0.01</td>
<td>−3 ± 1</td>
</tr>
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<td>STEs, PEG 5 kDa, R = 7%</td>
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<td>0.08 ± 0.02</td>
<td>−3 ± 1</td>
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<td>STEs, PEG 5 kDa, R = 72%</td>
<td>137 ± 2</td>
<td>0.16 ± 0.04</td>
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<td>In vivo study</td>
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<td>0.15 ± 0.01</td>
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<td>STEs, PEG 2 kDa, R = 72%</td>
<td>134 ± 3</td>
<td>0.15 ± 0.01</td>
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Table 1: PEG-modified and non-modified, loaded and non-loaded sterosome (STE) characterization: Hydrodynamic diameter (Z-Ave), polydispersity index (PdI) and zeta potential (ZP), used for complement activation, macrophage uptake and in vivo studies. STEs were modified using DSPE-PEG, whose PEG chain lengths were 2 or 5 kDa. R is the ratio DSPE-PEG/PA-Chol (w/w). (n = 3, mean ± SD).

Fig. 1. Sterosome (STE) surface modification. Variation of hydrodynamic diameter (Z-Ave) and zeta potential (ZP) vs. time when STEs were incubated at 37 °C with DSPE-PEG whose PEG chain lengths were 1 (A), 2 (B), 3 (C) and 5 kDa (D), at R = 7 (%) 14 (●), 36 (○) and 72% (□). R is the ratio DSPE-PEG/PA-Chol (w/w). (n = 3, mean ± SD).

a Non-dialyzed STEs. Other non-identified STEs were dialyzed (15 kDa membrane cut-off).
to be 350 µg/mL in order to reach a CH50 unit consumption of about 80%. No significant difference was observed between the different PEG chain lengths (2 or 5 kDa), and DSPE-PEG/PA-Chol ratio (7 or 72% (w/w)).

### 3.4. Sterosome stealth capacity in relation to macrophage uptake

The uptake of PEG-modified and non-modified STEs by human macrophages (THP-1 cell line) was measured at 4 and 37 °C, using flow cytometry (Fig. 5). At 4 °C, low fluorescence levels were observed in macrophages, whether the STEs were modified or not; the STEs were not captured by macrophages since phagocytosis is an energy-dependent process and is inhibited at low temperatures [19]. At 37 °C, a high fluorescence intensity was found inside the cells when non-modified STEs were incubated, reflecting an effective uptake. When STEs were PEGylated, the fluorescence
intensity inside cells significantly decreased (p < 0.01), indicative of a decreased macrophage uptake in comparison to non-modified STEs. The decrease was larger for a DSPE-PEG/PA-Chol ratio of 72% (w/w) compared to the levels observed for a ratio of 7% (w/w). No significant difference was observed between the PEG chain lengths (2 and 5 kDa).

3.5. Proof of concept of sterosome in vivo stealth capacity

The impact of PEG post-insertion on the nanocarrier circulation time after intravenous administration in nude SCID mice was determined by comparing DiD-loaded, non-modified and PEG-modified STEs (DSPE-PEG 2 kDa/PA-Chol ratio of 72% (w/w)). Images of entire animals were recorded 30 min (Fig. 6), 4, 8, 24, 48 and 96 h after STE injection (see supplementary information, Figs. S3 and S4). For non-modified STEs, a rapid accumulation was observed in the liver and spleen, 30-min post-administration. In addition, no nanocarrier were detected, by means of this analytical technique, in the blood circulation since the fluorescence level for the rest of the animal corresponded to the background observed in the control mice (Fig. 6C and D). With PEG-modified STEs, the nanocarriers were still seen in the blood circulation 30 min post-administration as revealed by the fluorescence signal detected over the entire animal body (Fig. 6C and D). No significant accumulation of labelled STEs could be observed in the liver and spleen. The PEG-modified STEs circulation lasted longer. For non-modified STEs, no fluorescence signal could be detected 4 h after the injection (see supplementary information, Figs. S3 and S4). PEG-modified STEs were cleared progressively as decreasing fluorescence intensities over the entire animal were observed up to 96 h post administration (see supplementary information, Figs. S3 and S4). In addition, a considerable accumulation of the dye in the intestine was observed after 4 h, where it remained detectable for up to 168 h (see supplementary information, Fig. S5).

4. Discussion

Sterosomes (STEs) provide a novel class of nanovesicles presenting distinct characteristics, including a high degree of impermeability (due to a high cholesterol molar fraction: 0.7) in comparison to phospholipidic liposomes, and hence high loading stability [8,10,16,20,21]. This formulation differs from niosomes and other non-phospholipidic liposomes found in the literature, due to a binary mixture of Chol and PA [22]. STEs were recently used for the first time for biological application. Cationic STEs (replacing PA with stearylamine as the monoalkylated amphiphile) were found efficient to target siRNA to adipose derived mesenchymal stem cells. The efficient down regulation of Noggin (specific antagonist of bone morphogenic protein) led to the potent osteogenic differentiation in vitro of the stem cells and promoted bone regeneration in a mouse calvarial defect model [23].

To ensure the long-term circulation after systemic administration, liposome surface must be decorated with polymer chains, and PEG is the most commonly used polymer for this purpose [24]. It was shown that PEG could be grafted to STE surface by a pre-insertion method, which involves adding the amphiphilic PEGylated molecule during the liposome preparation [10]. The major drawback for pre-insertion method is the double surface modification. The outer surface of the liposome is modified by the PEG chains, but so the inner surface. In some cases, this led to rapid liposome destabilization and problem for drug loading [11–13]. We showed here that, despite the high order of the apolar bilayer, the post-insertion method is applicable to STEs. DSPE-PEG was incubated with non-modified STEs and the post-insertion efficacy was monitored using ZP measurements. Non-modified STEs have a negatively-charged surface (ZP about –40 mV). During the kinetics of post-insertion, the ZP rapidly shifted to values close to neutrality as PEG at the STE surface screens the negative charges. This phenomenon was already observed for phospholipidic liposomes. For example, Nag et al. measured ZP values of –30 and

5. Conclusion
PEGylation of STES can be obtained using post-insertion, despite their high ordered bilayer consisted of PA and Chol. Some evidence observed after in vitro experiments: complement activation decrease and low macrophage uptake for PEG-modified STES, were confirmed after intravenous administration in animal model, where the PEG-modified STES were present for longer in the blood stream than the non-modified ones. A proof of concept was established: the post-insertion of PEG chain to STES produced stealth STES. Further systematic in vivo studies have to be carried out to find the best PEG-modified STES in terms of pharmacokinetic and biodistribution profiles, amongst all types of PEG-modified STES previously described, in order to improve therapeutic efficacy using active agents. STES appear to be a promising nanocarrier platform and the ability to improve their circulation period in the blood stream is a critical step in their future use in nanomedicine.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Acknowledgements
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Appendix A. Supplementary material
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2017.02.008.

References


