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Research paper

Long-circulating poly(ethylene glycol)-coated emulsions to target solid tumors

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Abstract

The purpose of this study was to develop oil-in-water emulsions (100–120 nm in diameter) and to correlate the surface properties of the emulsions with blood residence time and accumulation into neoplastic tissues by passive targeting. We investigated the effect of phospholipid and sphingolipid emulsifiers, hydrogenated soybean phosphatidylcholine (HSPC) and egg sphingomyelin (ESM), in combination with polysorbate 80 (PS-80) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE)-PEG lipids of various PEG chain lengths and structures in prolonging circulation time and enhancing accumulation into B16 melanoma or C26 colon adenocarcinoma. The relationship between amphiphile molecular packing at the air/water interface on emulsion stability upon dilution in albumin and circulation longevity *in vivo* was also explored for non-PEGylated emulsions. PEGylation of the droplet surface with 10–15 mol% of DSPE-PEG 2000 or 5000 enhanced the circulation time of the emulsions, however, accumulation was only observed in the C26 tumor model. The tighter molecular packing observed with ESM/PS-80 monolayers at the air/water interface compared to HSPC/PS-80 correlated with improved emulsion stability *in vitro*, however, enhanced circulation time *in vivo* was not observed. A better understanding of the relationships between composition and performance will result in improved emulsion-based drug delivery vehicles for cancer therapy.

Keywords: Long-circulating emulsions; Poly(ethylene glycol); Biodistribution; Pharmacokinetics; Drug carriers

1. Introduction

One of the major difficulties in cancer therapy is to achieve good specificity of antineoplastic agents for their intended site of action in the body. As a result of their toxicity towards healthy tissues, many anticancer drugs are often administered at doses that are subtherapeutic. Over the past three decades, a great deal of research has been devoted to altering the pharmacokinetic and biodistribution profiles of these drugs by encapsulating them in colloidal drug carriers such as liposomes [1], nanoparticles [2] and polymeric micelles [3]. These nanoscopic systems can enhance drug accumulation at the tumor site and reduce distribution to healthy tissues, provided that the encapsulated cargo remains associated with the carrier after intravenous (i.v.) injection [4,5]. One method in which drug carriers achieve this selectivity is by the enhanced permeation and retention (EPR) effect (also known as passive targeting), which exploits the difference in capillary structure between healthy and cancerous tissues [6]. Neoplastic tissues generally have porous or "leaky" vasculature and

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poor lymphatic drainage allowing for enhanced permeation of colloidal particles across the endothelium and greater retention within the tumor [7,8]. As a rule of thumb, the concentration of drug carrier in the blood should remain high for more than 6 h such that the vehicle can extravasate at the tumor site progressively over time [7]. However, after i.v. administration, colloidal drug carriers are usually recognized as foreign bodies and are rapidly taken up by circulating monocytes and macrophages in the liver, spleen and bone marrow. The ability of colloidal particles to evade the mononuclear phagocyte system (MPS) and exhibit long residence times in blood depends largely on carrier size and the physicochemical properties of the surface.

Modifying the colloid surface with a hydrophilic and flexible polymer such as poly(ethylene glycol) (PEG) is widely used to prolong circulation time [9,10]. The longevity of PEGylated colloids is attributed to the highly hydrated and flexible PEG chains, which reduce interactions with plasma proteins and cell surfaces [11,12]. Incorporating sphingomyelin (SM) at the interface is another approach that has been shown to enhance the circulation longevity of emulsions and liposomes [13,14]. For example, Takino et al. [13] reported that adding SM to an egg phosphatidylcholine (PC) emulsion prolonged circulation time and decreased uptake by the MPS organs. Similarly, Redgrave et al. [15] demonstrated that increasing the proportion of SM to PC further enhanced systemic residence time and resulted in a corresponding decrease in liver uptake. Moreover, liposomes composed of SM/cholesterol (Chol) (55/45, molar ratio) were shown to prolong the half-life of encapsulated vincristine, ciprofloxacin and vinorelbine [16–19]. In fact, this SM/Chol formulation with encapsulated vincristine was evaluated in Phase III clinical trials [20]. It has been proposed that SM confers circulation longevity to emulsions and liposomes by enhancing membrane rigidity, which increases membrane stability in the presence of serum [14,21–23].

Among the various drug delivery systems for cancer therapy, emulsions are promising carriers due to their biocompatibility, reasonable stability, ability to solubilize large quantities of lipophilic compounds and relative ease of manufacture at an industrial scale [24,25]. Despite the vast amount of literature available on emulsions as carriers for anticancer drugs, few studies have examined the relationships between composition and performance. An understanding of these relationships will allow for the formulation to be tailored to suit the delivery of specific drugs. Hence, the purpose of this study was to develop oil-in-water emulsions (100–120 nm in diameter) using pharmaceutically acceptable excipients and to correlate the properties of the emulsion surface with blood residence time and accumulation into neoplastic tissues by passive targeting. We investigated the influence of phospholipid and sphingolipid emulsifiers, hydrogenated soybean phosphatidylcholine (HSPC) and egg SM (ESM), in combination with polysorbate 80 (PS-80) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE)-PEG lipids of various PEG chain lengths and structures in prolonging circulation time and enhancing accumulation into solid tumors. The various formulations were evaluated in mice bearing subcutaneously implanted B16 melanoma or C26 colon adenocarcinoma. In addition, using the Langmuir balance technique, the relationship between amphiphile molecular packing at the air/water interface on emulsion physical stability and circulation longevity was explored for non-PEGylated emulsions.

2. Materials and methods

2.1. Materials

Hydrogenated soybean phosphatidylcholine (HSPC, >99% PC), ESM and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-monomethoxy-[PEG 2000] (DSPE-PEG 2000) were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). Tricaprylin (TC) and PS-80 were obtained from Sigma–Aldrich (Oakville, ON, Canada). DSPE-PEG 5000 was bought from Avanti Polar Lipids Inc. (Alabaster, AL) and DSPE-*N*-[(pentaerythritol polyoxyethylene) glutaryl] (DSPE-4-armPEG) (PEG MW 2000) was from the NOF Corporation (Tokyo, Japan). [^{3}H]Cholesteryl hexadecyl ether ([^{3}H]CHE, 51.0 Ci/mmol), Hionic-Fluor®, Soluene 350® and Solvable® were obtained from Perkin-Elmer (Woodbridge, ON, Canada). Sodium chloride (0.9% w/v) injection USP was purchased from B. Braun Medical Inc. (Irvine, CA). Bovine serum albumin (BSA) was supplied by Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). All products mentioned above were used without further purification. Chloroform and hexane (purification grade), purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada), were further purified by filtration through drying columns on a PureSolv system (Innovative Technology Inc., Boston, MA) and distilled prior to use. Ethanol was obtained from Commercial Alcohols Inc. (Brampton, ON, Canada) and distilled. Water was deionized with a MilliQ® purification system (Millipore, Bedford, MA).

2.2. Compression isotherms

Surface pressure–molecular area (π–A) isotherms of HSPC, ESM, PS-80, HSPC/PS-80 (1:3, w/w) and ESM/PS-80 (1:3, w/w) were measured with a Langmuir–Blodgett trough (300 × 200 × 5 mm) from Nima Technology (Coventry, England). Surface pressure was determined by means of a Wilhelmy plate attached to a microbalance. The subphase was a phosphate-buffered saline (PBS, pH 7.4) composed of 75 mM sodium chloride, 53 mM sodium phosphate dibasic and 13 mM sodium phosphate monobasic, filtered through a 0.22-μm membrane. The temperature of the subphase was maintained at 25 °C with a thermostated, circulating water bath. Solutions of HSPC and ESM were prepared at a concentration of 1 mg/mL in chlo-

roform and hexane:ethanol (95:5, v/v), respectively. Fifty microliters of solution was deposited dropwise at the air/water interface using a microsyringe. For PS-80, dissolved in chloroform (0.25 mg/mL), 20 μL of solution was spread on the subphase. In the case of the mixed monolayer isotherms (0.25 mg/mL total amphiphiles), 20 μL of HSPC/PS-80 (1:3, w/w) dissolved in chloroform and ESM/PS-80 (1:3, w/w) prepared in hexane:ethanol (95:5, v/v) were deposited. The films were compressed at a constant barrier speed of 8 cm^2/min after initial delay periods of 30 min and 1 h for lipids spread in chloroform and hexane:ethanol, respectively. To reduce contamination from the atmosphere, the entire apparatus was located in a closed Plexiglas cabinet.

From the π–A isotherms, monolayer compressibility at a given surface pressure (π) was calculated using Eq. (1):

$$C_s = (-1/A_\pi)(dA/d\pi)_\pi \quad (1)$$

where C_s is the modulus of compressibility and A_π represents the molecular area at a particular surface pressure.

2.3. Preparation and characterization of emulsions

The emulsions were prepared by probe sonication using a Sonic Dismembrator (model 550, Fisher Scientific, Pittsburgh, PA). Prior to sonication, TC, PS-80 and either ESM or HSPC were mixed together under magnetic stirring above the phase transition temperature of ESM and HSPC (ca. 55 °C) for 10 min. The dispersing phase (0.9% NaCl in water) was then added to the premix and the formulation was heated/mixed at 55 °C for an additional 20 min. The volume was adjusted with 0.9% NaCl in water until the lipid phase represented 9% (w/v) of the emulsion. After the premix step, the formulation was sonicated at medium intensity (80 W) for 40 s on pulse mode (pulse on, 2 s; pulse off, 0.2 s). The composition of oil and emulsifier was TC/PS-80/HSPC or ESM (5:3:1, w/w). PEGylated emulsions were prepared by incubating an aqueous micelle solution of DSPE-PEG with a preformed ESM emulsion for 1.5 h at 60 °C. The amount of DSPE-PEG added to the emulsions corresponded to either 10 or 15 mol% of the total surface components (excluding TC). [^{3}H]CHE, a non-exchangeable lipid derivative, was incorporated into each emulsion during emulsification to track the distribution of the entire droplet *in vivo*.

The mean hydrodynamic diameter and size distribution of the emulsions were determined by dynamic light scattering with a Malvern Autosizer (Malvern Instruments Ltd., Malvern, UK) at 25 °C and a fixed angle of 90°. Measurements were performed in triplicate after dilution of the emulsion in water.

The physical stability of the non-PEGylated HSPC and ESM emulsions upon dilution in 4% (w/v) BSA was characterized by calculating the percent change in mean hydrodynamic diameter before and immediately after incubation with albumin. The emulsions were incubated at 37 °C for various durations (30 min, 1 and 2 h).

2.4. Biodistribution studies

The *in vivo* experiments were performed on either C57BL/6 mice (female, 18–21 g) bearing B16–F10 melanoma or Balb/C mice (female, 18–21 g) with C26 colon adenocarcinoma. Animal care and handling were approved by the Animal Welfare and Ethics Committee of the University of Montreal in accordance with the Canadian Council on Animal Care guidelines. The hair on the back and hind legs of the mice were removed by shaving. The B16 and C26 cells were harvested by trypsinization, resuspended in growth medium and injected subcutaneously in three separate locations on the back of each mouse. For the B16 cells, approximately 1×10^7 cells in 50 μL of growth medium were delivered per implantation site, while 2×10^6 of C26 cells in 50 μL were injected. The formulations were administered when the tumor volume reached approximately 20 mm^3. The volume (V) of each tumor was calculated from Eq. (2):

$$V = 1/2(4\pi/3)(L/2)(W/2)H \quad (2)$$

where L is the length, W is the width and H is the height.

The emulsions were prepared as described above and diluted with NaCl (0.9%) for injection. The tumor-bearing mice were anesthetized with isoflurane and administered 5.4 mg of lipids/mouse (excluding DSPE-PEG) in a 100-μL injection volume via the subclavian vein. Each mouse received approximately 0.7 μCi [^{3}H]CHE.

The mice ($n = 4$–5 per group) were sacrificed at 2, 6 and 12 h post-injection. At each time point, the blood, tumor and organs of interest (liver, spleen, heart, lungs, kidneys and a piece of muscle) were extracted and assayed for [^{3}H]CHE content. Blood was sampled by cardiac puncture and placed into pre-weighed scintillation vials. Excess blood was removed from the organs by passing a continuous flow of 0.9% saline through the systemic circulation via the heart. The tumors and organs of interest were then harvested, wiped and weighed. The blood was digested in a mixture of isopropanol/Soluene® (1:1–1:3, v/v) or isopropanol/Solvable® (1:1–1:3, v/v) at 60 °C until complete solubilization. The samples were allowed to cool down to room temperature and were then discoloured with hydrogen peroxide (H_2O_2, 30% v/v) in aliquots of 100 μL. The organs and tumor were dissolved in Soluene® or Solvable® tissue solubilizers at 60 °C until total digestion. Hionic-Fluor scintillation cocktail (10 mL) was added to the solubilized tissues and the samples were stored overnight at 4 °C in the dark prior to counting. The amount of [^{3}H]CHE radioactivity in blood, organs and tumor was assayed using a Beckman liquid scintillation counter (model LS 6500, Beckman, Fullerton, CA). The quenching of radioactivity due to the digested tissues was corrected with a quench curve. The percent injected dose (ID) of emulsions in blood was determined by assuming that the

total mass of blood represents 7.2% of the mouse body weight [26]. Formulations were compared at each time point with a one-way analysis of variance followed by Fisher's post hoc test. The level of significance was a p value <0.05.

3. Results and discussion

3.1. Compression isotherms

The compressibility and molecular conformations of two high phase transition lipids, HSPC and ESM, were characterized at the air/water interface using the Langmuir balance technique. Isotherms of pure HSPC and ESM monolayers in PBS buffer (pH 7.4, 25 °C) are presented in Fig. 1a. For clarity, only one isotherm for each compound was selected from a series of three reproducible profiles. The ESM monolayer displayed a two-dimensional phase transition from liquid-expanded (chain disordered) to liquid-condensed (chain ordered). This transition region can be seen more clearly in Fig. 1b (at about 20 mN/m) by the change in slope of the π–A isotherm, expressed as the modulus of compressibility (see Eq. 1). The two-dimensional phase transition is consistent with previous reports, however, the sharpness of the transition region has been shown to vary depending on several factors including subphase temperature, chain length and heterogeneity in acyl chain composition [27,28]. Naturally occurring SMs such as bovine brain SM and ESM tend to broaden the transition region compared to pure 18:0 and 16:0 SM, which are the main lipids in bovine brain SM and ESM, respectively [27]. The broader transition is most likely due to the heterogeneity in acyl chain composition, which would affect the alignment/ordering of the chains. The HSPC film, on the other hand, did not undergo an order–disorder transition like ESM. Instead, HSPC shifted from gas directly into a highly ordered liquid-condensed phase and then to a collapsed regime (Fig. 1a). HSPC formed a less compressible film at the air/water interface as indicated in Fig. 1b by the lower modulus of compressibility compared to ESM at all surface pressures. Another important difference between the molecules is that ESM could form a more densely packed film at higher surface pressures. Indeed, above about 25 mN/m, the area occupied per molecule was smaller for ESM than HSPC (Fig. 1a).

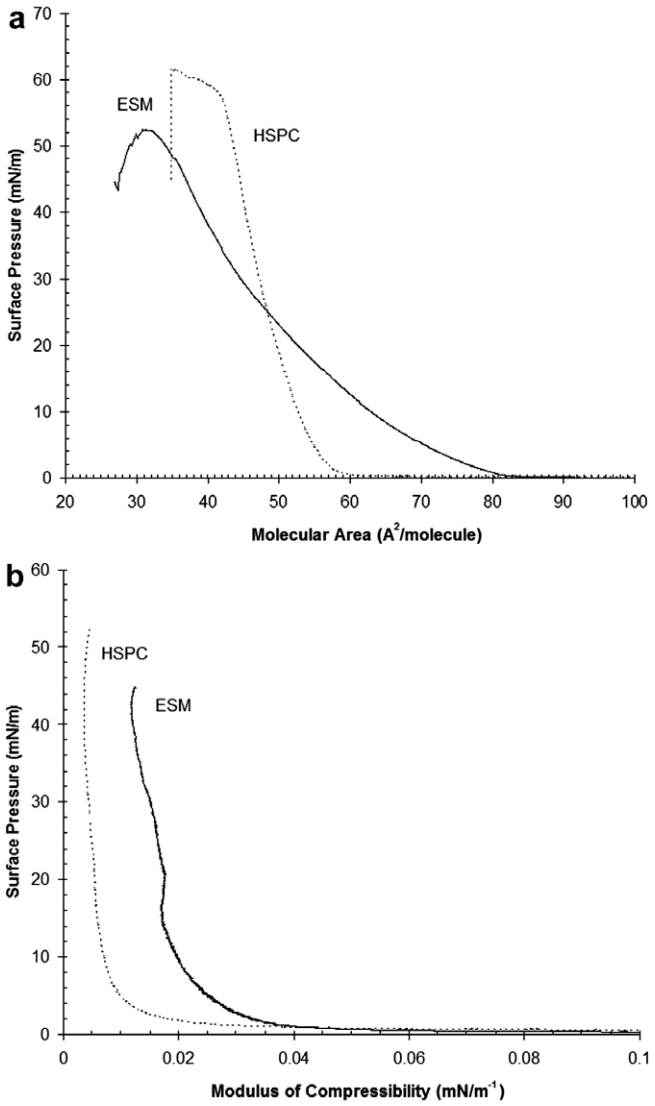

Fig. 1. Surface pressure versus molecular area (a) and surface pressure versus modulus of compressibility (b) plots of HSPC and ESM at the air/water interface. Subphase conditions: PBS, pH 7.4, 25 °C.

The differences between HSPC and ESM molecules at the air/water interface can be rationalized by their structural discrepancies. Both ESM and HSPC share a common zwitterionic phosphorylcholine headgroup, however, they differ in the degree of chain unsaturation and in their ability to form hydrogen bonds. HSPC, like other PCs, contains two acyl chains that are esterified to a glycerol backbone. These acyl chains are approximately the same length, however, unlike most naturally occurring PCs, HSPC contains no unsaturations (major component is 16:0, 18:0). For ESM, a saturated acyl chain is linked to a sphingosine through an amide bond. The greater compressibility of ESM may be due to the unsaturation present between carbons 4 and 5 of the sphingosine, which could produce a more disordered and fluid monolayer than HSPC. The HSPC molecules are more ordered in the hydrophobic region due to the long and completely saturated acyl chains and thus are less compressible in the liquid phase. These results are consistent with the findings of Smaby et al. [27], whereby ESM was more compressible than distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) (18:0, 18:0), a saturated PC with long acyl chains, at all surface pressures. ESM was also more compressible than 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphatidylcholine (PSPC) (16:0, 18:0) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) (16:0, 16:0) at surface pressures above 5 and 10 mN/m, respectively [27]. Below these surface pressures, PSPC and DPPC were in a chain-disordered regime and were slightly more compressible than ESM [27].

In addition to the differences in chain unsaturation, ESM has a much greater hydrogen bonding capacity than HSPC due to the amide and hydroxyl groups, which are not present in phospholipids. The intra- and intermolecular hydrogen bonding within and between ESM molecules may facilitate a more condensed organization of the head group region reducing the degree of hydration in the polar region and allowing for denser packing of the molecules upon compression compared to HSPC. These results are consistent with the findings of other group which report a lower degree of head group hydration in SM containing monolayers and bilayers compared to PCs [29–31].

The influence of PS-80, a major component in the emulsion, on the phase behavior of HSPC and ESM is illustrated in Fig. 2a. For all isotherms, the collapse pressure was not achieved. Consequently, the monolayers were compressed until the minimum area of the trough was reached. In the presence of PS-80, HSPC and ESM displayed gaseous and liquid-phase behavior over the range of molecular areas investigated. These mixed monolayers were more fluid and compressible than pure HSPC and ESM films. This is probably a result of the larger, more hydrated head group of PS-80 (poly(ethylene oxide) 20 sorbitan) which would occupy larger molecular areas than phosphorylcholine at the air/water interface. All three monolayers exhibited similar compressibility (Fig. 2b), however, ESM/PS-80 formed a more densely packed film as indicated by the smaller surface areas occupied by this mixture (Fig. 2a). A hypothesis proposed to explain this phenomenon is that ESM may reduce head group hydration by intra- and intermolecular hydrogen bonding within ESM molecules and between neighboring ESM molecules or with PS-80. As a result, the reduction in hydration of the head group region could permit denser packing of the molecules. In the context of injectable emulsions for drug delivery, incorporating ESM into a formulation emulsified with PS-80 may potentially enhance circulation time by the tighter molecular packing at the droplet surface. Owing to the interesting properties of ESM/PS-80 monolayers, we then investigated whether ESM could enhance the physical stability and circulation longevity of an emulsion coemulsified with PS-80.

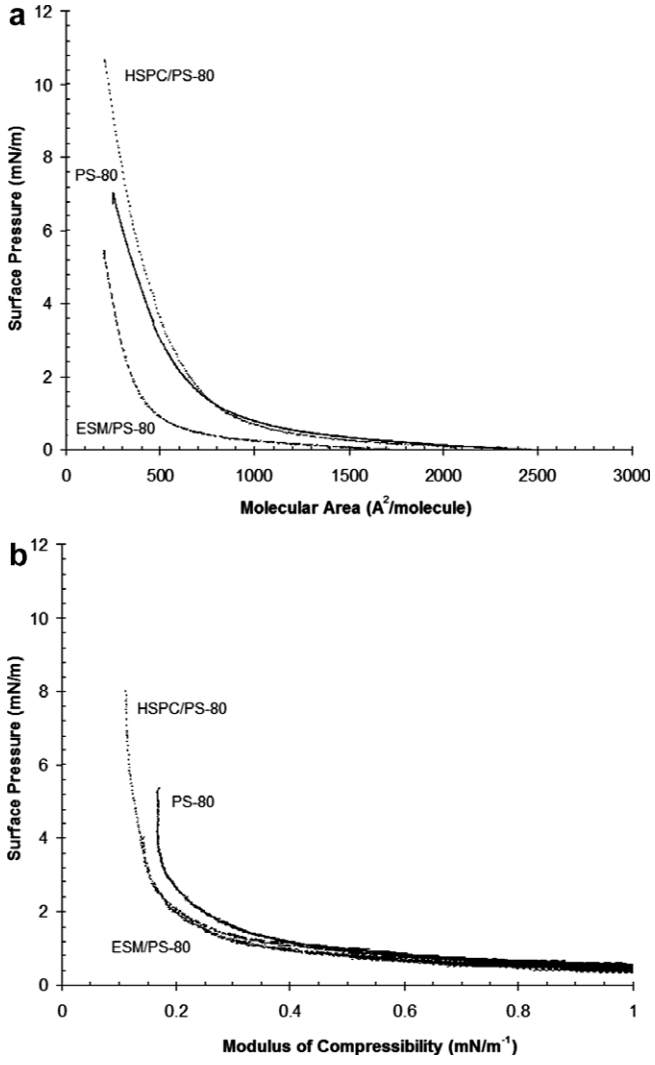

Fig. 2. Surface pressure versus molecular area (a) and surface pressure versus modulus of compressibility (b) plots of PS-80, HSPC/PS-80 (1:3, w/w) and ESM/PS-80 (1:3, w/w) at the air/water interface. Subphase conditions: PBS, pH 7.4, 25 °C.

3.2. Emulsion characterization

Oil-in-water emulsions with mean diameters ranging from 100 to 120 nm were prepared by probe sonication (Table 1). The internal phase consisted of TC and was emulsified with a combination of PS-80 and either HSPC or ESM. The weight ratio of oil and emulsifier was kept constant at 5:3:1 (TC/PS-80/HSPC or ESM). TC was chosen as the internal phase because medium chain triglycerides are generally better solubilizers for drugs than long chain triglycerides and thus these emulsions could potentially be used to encapsulate drugs [32]. PS-80 was the main emulsifier since it is well tolerated for i.v. application and the high density of short PEG segments at the emulsion interface may extend systemic circulation [33,34]. The physical stability of non-PEGylated HSPC and ESM emulsions upon dilution in 4% (w/v) BSA is presented in Table 2. The results showed that emulsions prepared with HSPC were considerably less stable in the presence of albumin compared to those coemulsified with ESM. The superior stability observed with ESM emulsions may be attributed to a tighter monolayer at the interface, which could reduce protein adsorption onto the emulsion surface, thereby preventing the destabilization of the interface.

Only emulsions prepared with ESM were incubated with various DSPE-PEG derivatives. The final concentration of DSPE-PEG in the formulation represented 10–15 mol% of the total surface components (excluding TC). The presence of DSPE-PEG did not considerably alter the mean size of the emulsion (Table 1). The physical stability of the emulsions upon storage was assessed at ambient conditions. We observed that the emulsions, with or without PEGylation,

Table 1
Properties of the lipid emulsions administered to mice bearing either B16–F10 melanoma or C26 colon adenocarcinoma

Formulation[a]	PEG content (mol%)[b]	Mean diameter (nm)	PDI	Tumor
TC/PS-80/HSPC	–	113	0.27	B16–F10
TC/PS-80/ESM	–	103	0.32	B16–F10
TC/PS-80/ESM/DSPE-PEG 2000	10	107	0.25	B16–F10
TC/PS-80/ESM	–	110	0.29	C26
TC/PS-80/ESM/DSPE-PEG 2000	10	114	0.26	C26
TC/PS-80/ESM/DSPE-PEG 2000	15	103	0.23	C26
TC/PS-80/ESM/DSPE-PEG 5000	10	121	0.21	C26
TC/PS-80/ESM/DSPE-4-armPEG	10	110	0.24	C26

[a] The weight ratio of lipids and surfactant except for DSPE-PEG was kept constant (TC/PS-80/X = 5:3:1, w/w, where X is either HSPC or ESM).
[b] Expressed as mol% of total surface components (excluding TC).

Table 2
Stability of non-PEGylated emulsions after incubation with 4% (w/v) BSA

Formulation[a]	Dispersed phase concentration (mg/mL)	Percent change in mean droplet size[b]		
		30 min (%)	1 h (%)	2 h (%)
TC/PS-80/HSPC	3	24 ± 11	19 ± 5	17 ± 4
	0.5	29 ± 4	13 ± 3	14 ± 10
TC/PS-80/ESM	3	<4	<4	<4
	0.5	<4	<4	<4

Mean ± SD ($n = 3$).
[a] The weight ratio of lipids and surfactant is TC/PS-80/X = 5:3:1, w/w, where X is either HSPC or ESM.
[b] After incubation with 4% (w/v) BSA at 37 °C in PBS, pH 7.4.

increased in size by about 20–40% in 1 month and eventually phase separated after 3–4 months (data not shown). As a result of the limited physical stability, the emulsions were freshly prepared for each *in vivo* study.

3.3. Biodistribution studies

The influence of ESM and DSPE-PEG 2000 in prolonging the circulation time of emulsions was first assessed in C57BL/6 mice inoculated with B16 melanoma. To track the distribution of the droplets *in vivo*, the emulsions were labeled with [^{3}H]CHE. This marker has been commonly used for biodistribution studies since it is highly lipophilic and non-exchangeable [35,36]. In this first experiment, three different formulations were evaluated: TC/PS-80/HSPC, TC/PS-80/ESM and TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000. As shown in Fig. 3a, the non-PEGylated HSPC and ESM emulsions were quickly removed from the systemic circulation with less than 10% of the ID remaining in blood after 2 h. Emulsions containing ESM circulated slightly longer in blood than HSPC, however, statistical significance could not be demonstrated ($p > 0.05$). The slightly higher residence time for the ESM emulsion may be attributed to a tighter monolayer at the interface, which could potentially enhance the stability of the emulsion in blood. This tighter monolayer, however, was insufficient to provide significant enhancement in circulation longevity. As anticipated, Fig. 3a shows that PEGylation of the TC/PS-80/ESM emulsion with 10 mol% PEG 2000 significantly prolonged circulation time ($p < 0.05$). Two hours after administration, approximately 35% ID was still present in blood. Surprisingly, despite the long-circulating properties of the PEGylated emulsion, all formulations showed similar accumulation into B16 melanoma tissues (Fig. 3b). After 12 h, uptake into the tumor was between 1.2% and 2.2% ID/g of tissue. The comparable accumulation into the tumor for short and long-circulating emulsions may be a result of low pore cut-off size in the tumor vasculature, restricting droplet extravasation across the vessel wall. Depending on the cell line, tumors can have varied pore cutoff sizes, ranging from 200 to 1.2 μm [37]. Despite the limited distribution into B16 melanoma, all emulsions accumulated more in the tumor than in the muscle (Figs. 3b and c). Our results for droplet distribution into the tumor are lower compared to long-circulating liposomes (~100 nm in diameter) whereby accumulation into B16 melanoma (inoculated intramuscularly or in the hind footpad) was between 4.5% and 13.2% ID/g 24 h after injection [38]. The lower uptake of our PEGylated emulsions into B16 melanoma may be due to differences in the site of tumor inoculation, the size of the tumor at the time of injection and the time point at which the tumor was harvested. It was found that tumor uptake increased 1.7-fold, when liposome diameter was reduced from 138 to 97 nm without any significant change in blood residence time, demonstrating the importance of size on colloid extravasation into B16 melanoma [38].

The distribution of the emulsions to the liver, spleen, lungs, heart and kidneys after 2, 6 and 12 h post-i.v. injection is shown in Fig. 4. As expected, the emulsions distributed mainly to the MPS organs, with the majority of the formulation accumulating in the liver. PEGylation of the emulsions significantly reduced liver uptake at all time points. Distribution to the liver was comparable for both non-PEGylated emulsions prepared with either HSPC or ESM. For all emulsions, accumulation into the lungs, heart and kidneys was low.

The effect of DSPE-PEG 2000 content (10 and 15 mol%) and different DSPE-PEG derivatives in prolonging circulation time was then explored in Balb/C mice inoculated with C26 colon adenocarcinoma. The tumor model was changed as a result of the poor uptake of the long-circulating PEGy-

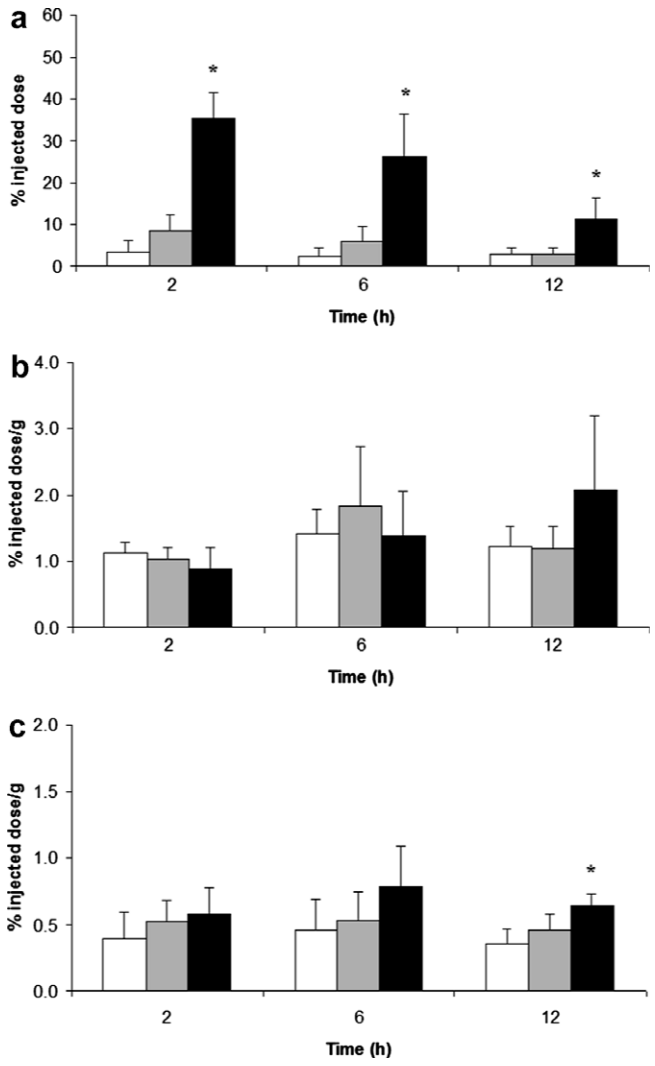

Fig. 3. Elimination profile of emulsions from blood (a) and distribution to B16–F10 melanoma (b) and muscle (c) after i.v. injection in C57BL/6 mice. Each mouse was administered 5.4 mg of lipids (excluding DSPE-PEG) in a 100-μL injection volume. Mean + SD ($n = 4$–5 mice/group). TC/PS-80/HSPC (□), TC/PS-80/ESM (▨) and TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000 (■). Statistically significant differences between plain and PEGylated emulsions are indicated. $^{*}p < 0.05$.

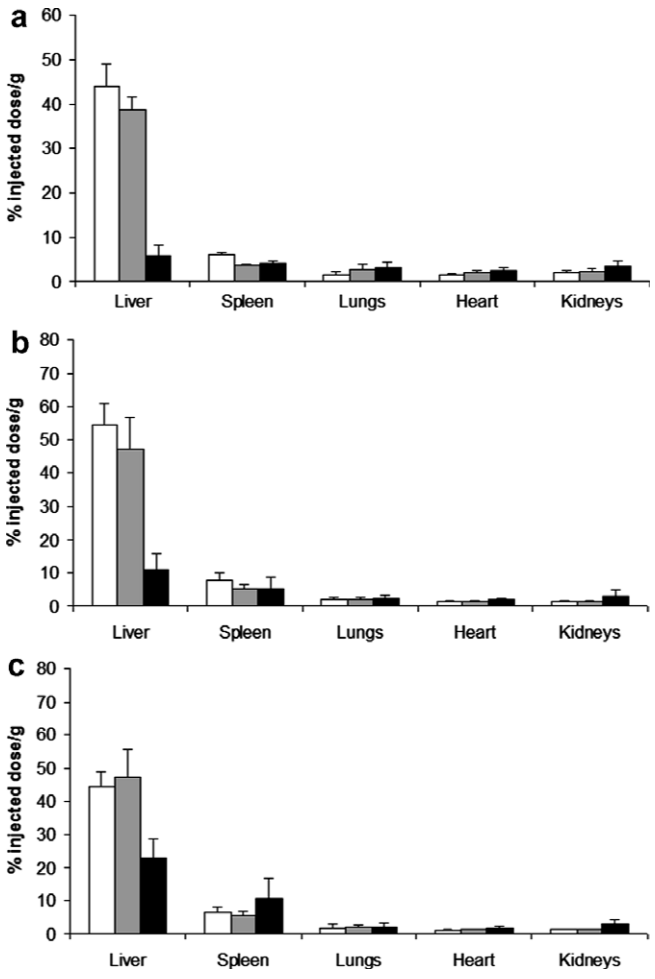

Fig. 4. Tissue distribution of emulsions after 2 h (a), 6 h (b) and 12 h (c) post-i.v. injection in C56BL/6 mice inoculated with B16–F10 melanoma. Mean + SD ($n = 4$–5 mice/group). TC/PS-80/HSPC (□), TC/PS-80/ESM (▨) and TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000 (■).

lated emulsions by the B16 tumor. In this study, five different formulations were studied: TC/PS-80/ESM, TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000, TC/PS-80/ESM/(15 mol%)DSPE-PEG 2000, TC/PS-80/ESM/(10 mol%) DSPE-PEG 5000 and TC/PS-80/ESM/(10 mol%)DSPE-4-armPEG. Elimination profiles of the emulsions from blood are shown in Fig. 5a. Comparable to the study in mice bearing B16 tumors, the non-PEGylated ESM emulsion was quickly removed from blood with less than 20% of the ID remaining in the circulation 2 h post-injection. Also similar to the mice inoculated with B16 melanoma, the emulsions containing 10 mol% PEG 2000 prolonged circulation time with about 40% of the ID still present in the blood after 2 h. Increasing the concentration of PEG 2000 to 15 mol% or enhancing the length of the PEG chain to 5000 g/mol did not further prolong blood residence time. It is possible that at 10 mol% PEG 2000, the colloid reached its optimum protection with PEG and any additional increase in concentration and chain length would not enhance circulation time further. Previous studies by our group have shown that at low PEG concentrations (less than 4 mol%), small increases in PEG concentration or increasing chain length from 2000 to 5000 g/mol enhanced the circulation time of lipid nanocapsules [35]. However, at 6 mol% and above, almost no difference was observed with increasing PEG 2000 concentration [35,39] or PEG chain length (2000–5000 g/mol) [35]. Similarly, Liu et al. [33] observed that increasing the concentration of PEG 2000 at the interface prolonged circulation time until a plateau in blood concentration was reached after approximately 5 mol%.

Even though grafting density was high (10 mol%), droplets coated with DSPE-4-armPEG did not circulate as long as the other single chain DSPE-PEG derivatives (Fig. 5a). The PEG chain lengths of each arm were probably too short to provide sufficient protection against opsonization. Indeed, the number of repeating ethylene oxide units was

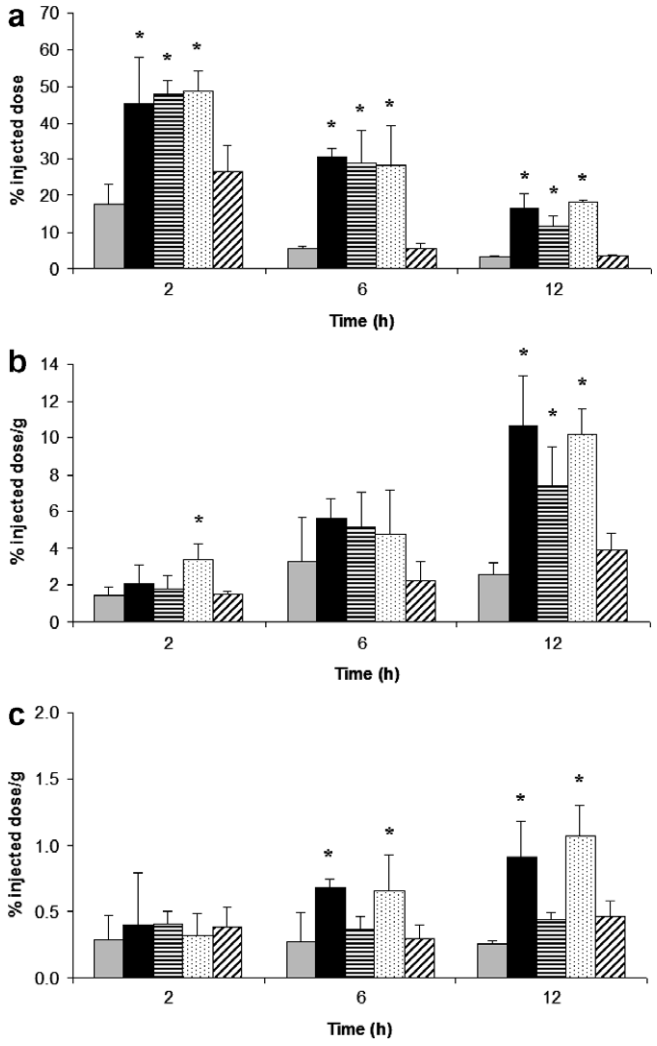

Fig. 5. Elimination profile of emulsions from blood (a) and distribution to C26 colon adenocarcinoma (b) and muscle (c) after i.v. injection in Balb/C mice. Each mouse was administered 5.4 mg of lipids (excluding DSPE-PEG) in a 100-μL injection volume. Mean + SD ($n = 4$–5 mice/group). TC/PS-80/ESM (▣), TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000 (■), TC/PS-80/ESM/(15 mol%)DSPE-PEG 2000 (▤), TC/PS-80/ESM/(10 mol%) DSPE-PEG 5000 (▩) and TC/PS-80/ESM/(10 mol%)DSPE-4-armPEG (◪). Statistically significant differences between plain and PEGylated emulsions are indicated. $^{*}p < 0.05$.

only 11 per arm for 4-armPEG (equivalent to about 500 g/mol per arm) compared to 45 and 113 for PEG 2000 and PEG 5000, respectively. Previous groups have shown that the circulation time of the carrier is strongly dependent on PEG chain length [40,41]. For example, Allen et al. [41] observed that liposomes grafted with PEG 1900 and PEG 5000 prolonged the blood residence time of the carrier, while liposomes coated with shorter chain PEG-lipid derivatives (i.e. PEG 750 and PEG 120) were removed more quickly from the systemic circulation.

Enhanced circulation time of PEGylated emulsions in blood translated into higher accumulation into C26 tumors (Fig. 5b). The distribution of emulsions grafted with PEG 2000 or PEG 5000 into the tumor was between 7.4% and 10.6% ID/g after 12 h, while only 2.6% and 3.9% ID/g was detected in the tumor for plain and 4-armPEG-coated emulsions, respectively. Even though plain and 4-armPEG-coated emulsions extravasated less into C26 tumors, all emulsions displayed selectivity for the tumor over the muscle. Indeed, no more than 1.1% ID/g was distributed to the muscle (Figs. 5b and c). Our results are in general agreement with those obtained with stealth liposomes, whereby their accumulation into subcutaneously implanted C26 colon adenocarcinoma after 1 and 16 h post-i.v. injection was approximately 7% and 18% ID/g, respectively [42]. These liposomes (~90 nm in diameter) reached a maximum accumulation of about 20% ID/g at the 24 h time point [42].

Regardless of the similar circulation times of emulsions with 10 mol% PEG 2000, accumulation was greater in C26 tumors compared to B16 at all time points. The greatest difference was detected at 12 h post-injection, whereby the emulsion exhibited a 5-fold greater accumulation into

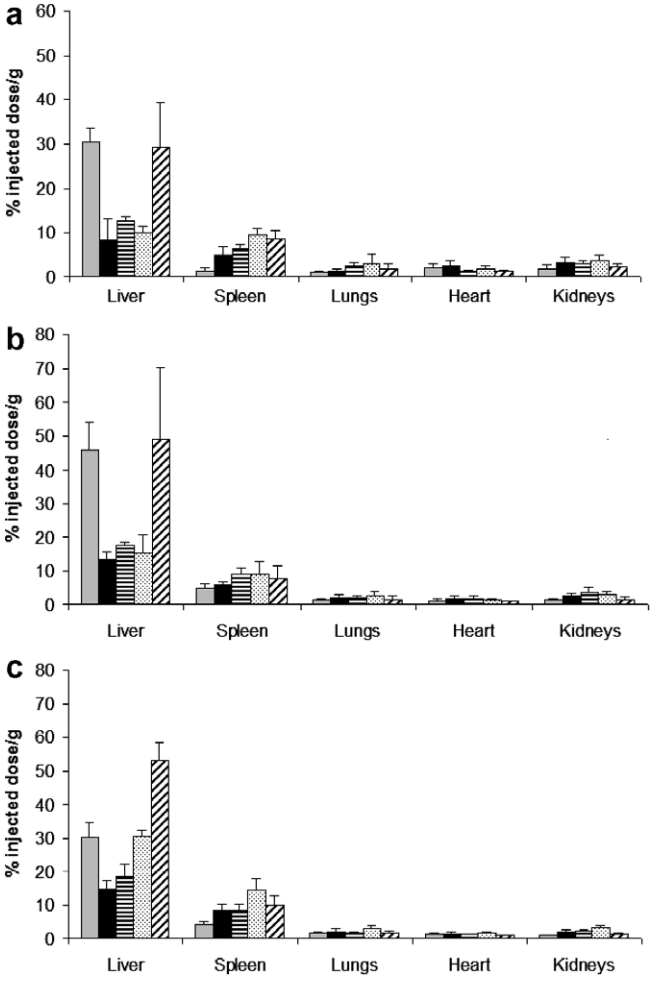

Fig. 6. Effect of DSPE-PEG derivatives on the tissue distribution of emulsions in Balb/C mice inoculated with C26 colon adenocarcinoma after 2 h (a), 6 h (b), and 12 h (c) post-i.v. injection. Mean + SD ($n = 4$–5 mice/group). TC/PS-80/ESM (▣), TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000 (■), TC/PS-80/ESM/(15 mol%)DSPE-PEG 2000 (▤), TC/PS-80/ESM/(10 mol%) DSPE-PEG 5000 (▩) and TC/PS-80/ESM/(10 mol%)DSPE-4-armPEG (◪).

C26 tumors than B16 (Figs. 3b and Fig. 5b). This observation may be explained by lower vascular permeability of the B16 tumor compared to C26. In addition to leaky vasculature, other factors such as differences in blood vessel density, blow flow rate and interstitial pressure in different tumor types can affect particulate accumulation [8]. Our finding is contrary to the results of Ishida et al. [43] who reported that B16 and C26 tumors showed similar permeability to liposomes with mean diameters ranging from 60 to 400 nm. The discrepancy between the two studies may be attributed to differences in vascular permeability and tumor volume at the time of injection.

The uptake of the emulsions by different organs after 2, 6 and 12 h post-i.v. injection is presented in Fig. 6. Similar to the study in C57BL/6 mice inoculated with B16 melanoma, the emulsions distributed mainly to the liver and accumulation into the lungs, heart and kidneys was low. The enhanced blood levels of emulsions with PEG 2000 (10 and 15 mol%) and PEG 5000 translated into lower uptake by the liver. In contrast, the plain and 4-arm-PEG-coated emulsions which were cleared from blood faster accumulated more in the liver.

Several studies have reported the ability of emulsions to enhance the accumulation of anticancer agents into solid tumors compared to the free drug [5,44,45]. While other reports have shown a greater reduction in tumor volume over time when drugs are encapsulated into long-circulating emulsions versus free drug [46–49]. However, despite the vast amount of literature on emulsions as drug carriers in cancer therapy not much work has been devoted to characterizing the accumulation of the droplet itself into solid tumors. In this study, we demonstrated that nanosized PEGylated emulsions prepared with commonly used pharmaceutical excipients can passively target neoplastic tissues. The degree of emulsion accumulation into the tumor was dependent on the PEG coating and tumor type, whereby C26 colon adenocarcinoma was more permeable to the emulsions than B16 melanoma. Moreover, a relationship between tighter molecular packing at the air/water interface and enhanced physical stability in the presence of albumin was established. However, prolonged circulation time *in vivo* was not observed.

The emulsions developed in this study can potentially enhance the accumulation of lipophilic anticancer drugs in tumor tissues and increase the therapeutic index for non-hepatotoxic drugs.

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