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Doubly radiolabeled liposomes for pretargeted radioimmunotherapy

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Abstract

The aim of this study was to design liposomes as radioactivity carriers for pretargeted radioimmunotherapy with favorable pharmacokinetic parameters. To monitor the liposomes integrity *in vivo*, their surface was radiolabeled with indium-111 bound to DTPA-derivatized phosphatidylethanolamine (DSPE-DTPA) and the aqueous phase was labeled by using an original active loading technique of radioiodinated Bolton–Hunter reagent (BH) that reacts with pre-encapsulated arginine to form a positively charged conjugate (^{125}I-BH-arginine). Different formulations of doubly radiolabeled liposomes were tested *in vitro* and *in vivo* to evaluate radiolabeling stability, integrity of the vesicles and their pharmacokinetics.

Radiolabeling yields were high (surface >75%, encapsulation >60%) and stable (>85% after 24 h in serum 37 °C). *In vivo*, the pharmacokinetic behavior of doubly radiolabeled liposomes was strongly dependant on the formulation. Blood clearance of PEGylated liposomes (DSPC/Chol/DSPE-DTPA/DSPE-PEG5%) was 0.15 mL/h compared to a conventional formulation (DSPC/Chol/DSPE-DTPA: clearance 1.44 mL/h). Non-encapsulated BH-arginine conjugate was quickly eliminated in urine (clearance 6.04 mL/h). Blood kinetics of the two radionuclides were similar and radiochromatographic profiles of mice serum confirmed the integrity of circulating liposomes. The significant reduction of activity uptake in organs after liposome catabolism (liver and spleen), achieved by the rapid renal elimination of ^{125}I-BH-arginine, should bring significant improvements for targeted radionuclide therapy with sterically-stabilized liposomes.

Keywords: Liposomes; Radiolabeling; Bolton–Hunter; Pharmacokinetics; Biodistribution

1. Introduction

Liposomes have shown promise as vehicle for targeted drug delivery in a range of clinical applications including cancer therapy. Radiolabeled liposomes have also potential applications in diagnostic imaging and radionuclide therapy. They have been labeled with technetium-99 m, indium-111 or gallium-67 and have shown an interesting tropism for tumors and infectious or inflammatory sites (Woodle et al., 1993). Specific tumor targeting of liposomes by coupling antibodies to the liposome surface (immunoliposomes) has been proposed a long time ago (Barbet et al., 1981; Connor et al., 1985). Recently pretargeting methods using bispecific antibodies (Cao et al., 2000) or the avidin–biotin system (Xiao et al., 2002) have been described. These multi-steps strategies provide an alternative way to specifically target liposomes to cancer cells. They should further improve the delivery of liposomes to tumors *in vivo* and renew the interest for liposome labeling with high activity doses of therapeutic radionuclides (Utkhede et al., 1994; Bao et al., 2003; Sofou et al., 2004). However, the ability of immunotargeted liposomes to deliver high doses of radioactivity to tumor cells *in vivo* remains to be demonstrated, partly because it is difficult to include all necessary features, i.e. long circulation times, stable radiolabeling with high activities and efficient antibody targeting, in the liposome preparation.

There is a considerable amount of data in the literature on the effect of liposomal composition on their pharmacokinetics and biodistribution (Allen and Hansen, 1991; Klibanov et al., 1991; Gabizon and Papahadjopoulos, 1992), but it is important to distinguish between the fate of the liposome envelope and of its content to understand which characteristics (stability, labeling or targeting techniques) must be altered to improve the liposome targeting efficiency.

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Until now, methods that have been described for labeling liposomes do not afford high specific activities and it is difficult to know whether small unilamellar vesicles (SUV) are taken up or circulate intact in the different tissues after intravenous injection. Liposomes have a bi-layer lipid membrane and it is easy to radiolabel this membrane but more difficult to obtain high specific activities. In this work, to monitor the liposome integrity *in vivo*, the surface was radiolabeled by binding indium-111 to DTPA-derivatized phosphatidylethanolamine (DSPE-DTPA) and the internal aqueous phase of the liposome was radiolabeled with a water-soluble small molecular weight tracer. To this effect, but also to make the liposomes carry an activity suitable for tumor therapy, we have developed a new radiolabeling method. The principle is similar to the active-loading of anthracyclines (Gabizon et al., 1998) and consists in encapsulating a radio-halogenated compound into preformed liposomes to obtain high specific activity (Mougin-Degraef et al., 2006). Active-loading methods, based on the ability of weak acids or bases to cross the liposome membrane as uncharged species (Clerc et al., 1995), have been applied to many substances (Madden et al., 1990; Cullis et al., 1997; Hwang et al., 1999) by creating pH gradients between the inside of the liposomes and the external medium. In this study, a slightly different method was used. Radioactivity is encapsulated into preformed liposomes by using a chemical reaction that transforms a neutral radio-iodinated molecule, capable of crossing liposome membranes, into a charged, water soluble compound, which remains entrapped in the liposome aqueous core. Briefly, the Bolton–Hunter (BH) reagent is labeled with iodine-125 and encapsulated in preformed liposomes containing high concentrations of arginine. Reaction of the activated ester with arginine creates a positively charged compound that remains entrapped in the liposome independently of a pH gradient.

Then, different formulations of doubly radiolabeled liposomes were evaluated in mice after radiolabeling stabilities *in vitro*. Pharmacokinetic parameters were determined for the two tracers (indium-111 and iodine-125) and an HPLC control of mice serum permitted us to compare the radiochromatographic profiles corresponding to the two radiotracers in order to check the integrity of the vesicles. Results of this study allowed us to evaluate the feasibility of solid tumor pretargeting with doubly radiolabeled and long-circulating liposomes.

2. Materials and methods

2.1. Materials

N-Succinimidyl-3-(4-hydoxyphenyl)-propionate (Bolton–Hunter reagent, BH) was purchased from Pierce Chemical Co. (Rockford, USA). Chloramine T, 1,2-dimyristoyl-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-glycero-3-phosphocholine (DSPC), cholesterol (Chol) and egg L-α-phosphatidylcholine (EPC) were from Sigma–Aldrich (Steinheim, Germany).

1,2-Dimiristoyl-sn-glycero-3-phosphoethanolamine-*N*-(methoxy-(polyethyleneglycol-2000)) (DMPE-PEG2000) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-(methoxy-(polyethyleneglycol-2000)) (DSPE-PEG2000) were from Avanti Polar Lipids (Alabaster, AL, USA) such as the manual extruding device used for downsizing the liposomes. DMPE-DTPA and DSPE-DTPA were synthesized in our laboratory.

^{111}In-indium chloride was from Mallinckrodt (Petten, The Netherlands) and ^{125}I-iodide sodium was purchased from Perkin-Elmer (Wellesley, MA, USA).

2.2. Liposomes preparation and characterization

Large unilamellar vesicles LUVs composed of EPC/Chol (66:34 molar ratio), DMPC/Chol/DMPE-DTPA or DSPC/Chol/DSPE-DTPA (68:30.5:1.5 molar ratio) were prepared according to the lipid film hydration method described by Bangham et al. (1965) followed by extrusion. Typically, 13.5 μmol of phospholipids, 6.6 μmol of cholesterol and 0.3 μmol of phospholipids coupled to the chelating agent were dissolved in chloroform/methanol (9:1 v/v) in a 10 mL round bottom flask. DMPE-PEG2000 or DSPE-PEG2000 (5 mol% or 8 mol% unless otherwise noted) was included in the preparation. A thin dry film of lipids was obtained by evaporation of the solvents in a rotary evaporator (Rotavapor®, Buchi). Hydration of the dry lipid film was accomplished by addition of 1 mL of aqueous phase and maintained above the gel-crystal transition temperature of the lipids during all the hydration procedure. To this effect, the flask containing the liposome suspension was mixed during 2 h on a rotary evaporation system without vacuum, at room temperature for conventional liposomes (DMPC/Chol or DSPC/Chol), and 50 °C or 74 °C for PEGylated liposomes containing respectively DMPE-PEG2000 or DSPE-PEG2000. Typically, the final concentration of the liposome suspension was 20 μmol of lipids per mL of aqueous phase.

To obtain small and homogeneous vesicles, the liposome suspension was sonicated for 2 min in a bath-type sonicator then repeatedly extruded through Nucleopore polycarbonate filters using a manual thermostat-heated extrusion device (Avanti® Polar Lipids, Alabaster, AL, USA). The suspension was filtered 20 times through filters with a pore size of 100 nm, at room temperature for conventional liposomes and at 50 °C or 74 °C for PEGylated liposomes, in a thermostat-heated extrusion device (Hope et al., 1985). A 100 nm filter was chosen because some authors have related that PEGylated liposomes with 120 nm average diameter showed the highest accumulation into the solid tumor (Ishida et al., 1999; Maeda et al., 2000). The size and polydispersity of the vesicles were determined by dynamic laser light-scattering measurements using a Malvern High Performance Particle Sizer (HPPS-ET, Instrument SA, UK). Measurements were performed in triplicate after dilution of the suspension in water. The mean sizes were 101 ± 2 nm (polydispersity index <0.1) for conventional liposomes and 127 ± 3 nm (polydispersity index <0.1) for PEGylated liposomes with 5% or 8% of PEG2000.

2.3. Radiolabeling procedure

For the chemical entrapment of the iodinated reagent, the dry lipid film (20 μmol lipids) was hydrated with 1 mL of

arginine solution (80 mmol/L in 4-(2-hydroxyethyl)-piperazine-1-sulphonic acid, HEPES 80 mmol/L pH 8.0) in order to obtain a physiological osmolality. Untrapped arginine was removed by size-exclusion chromatography on a Superdex® 200 column (Amersham Pharmacia Biotech, Orsay, France) eluted in 150 mmol/L, pH 5.6 phosphate buffer.

Bolton–Hunter reagent was labeled with iodine-125 by the chloramine-T method and purified by solvent extraction (Bolton et al., 1973). Briefly, to 2 μL of a fresh BH solution in anhydrous dioxan (0.5 mg/mL) were added 50 μL chloramine T (4 mg/mL in phosphate buffer) and 3.7 MBq to 37 MBq (0.1–1.0 mCi) of sodium ^{125}I-iodide. Incubating for approximately 15 s carried out the iodination, and then 400 μL of 100 mmol/L phosphate buffer (pH 7.4) was added. In order to avoid ester hydrolysis, the radiolabeled BH was immediately extracted with 500 μL of toluene. The organic phase was removed and transferred into a glass tube. The radiochemical purity was checked by thin layer chromatography using Silica gel 60 F_{254} (Merk, Germany) with chloroform/ethyl-acetate (1:1 v/v) and revealed with a phosphor-imager (445SI Molecular Dynamics, Amersham Pharmacia Biotech, France). The organic solvent was then evaporated using a dry nitrogen stream before adding the liposome suspension.

For the encapsulation of the ^{125}I-BH, 0.5 mL of arginine-containing liposomes, in phosphate buffer (0.15 mmol/L, pH 5.6), was added to the dry ^{125}I-BH reagent in a glass tube (90 nmol of reagent for 1 μmol total lipids). For the membrane radiolabeling with ^{111}In, citrate buffer (100 mmol/L, pH 5.0) was added in order to obtain a final citrate concentration of 10 mmol/L and a pH between 5 and 6. The $^{111}In\ Cl_3$ solution was added to the buffered liposomes preparation (until 44 MBq/μmol total lipids) and the suspension was incubated for 30 min at 37 °C. Then, a non-radioactive indium chloride solution (7.8 μmol/mL in HCL 0.02N) was added to saturate all DTPA groups present at the liposome surface. The labeling efficiencies were determined by counting the liposome suspension before and after chromatography on a PD-10 column with a γ-counter (Wallac 1480-Wizard® 3, Perkin-Elmer, Paris, France).

2.4. In vitro radiolabeling stability

In vitro radiolabeling stability of doubly radiolabeled liposomes was measured by incubating the liposome suspension, conventional or PEGylated, in phosphate buffered isotonic saline pH 7.4 (PBS) at 4 °C and in human serum at 37 °C (0.02 μmol to 2 μmol lipids/mL serum). At selected time intervals (from 0 h to 144 h), the liposomes were separated from free radioactivity by gel filtration chromatography using a PD-10 column, eluted in PBS.

2.5. Animal experiments

2.5.1. Pharmacokinetics and biodistribution

Various formulations of doubly radiolabeled liposomes were injected as an intravenous bolus injection via the tail vein of female BALB/c mice (mean body weight 20 g). Mice (4 per group) received 500 nmol of total lipids/mouse in 100 μL of PBS, containing 1.11 MBq (30 μCi) of total radioactivity (∼0.5 MBq of each radionuclide). At selected times post-injection (5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 24 h), blood samples were collected by eye sinus puncture, weighted and analyzed for ^{125}I and ^{111}In radioactivity in a gamma counter. The total radioactivity in the blood was determined by assuming that the total volume of blood was 7% of the mice body weight (Wu et al., 1981). Mice were sacrificed at 24 h by cervical dislocation and after dissection, normal organs and tissues were dried, weighted and counted in a gamma counter calibrated for the two isotopes. Results are expressed as the percentage of the total administered liposome dose accumulated per gram of tissue (% ID/g). Values are expressed as mean ± S.D. Standards of the injected material were made in duplicate and used to calculate the total injected dose and to correct for decay of the radioisotopes. Urines of mice placed in metabolic cages were collected and counted as described before. Results are expressed as the percentage of the total administered liposome dose (% ID).

2.5.2. Mice serum analysis

For *in vivo* serum stability, blood samples were collected from replicate mice at 1 h, 5 h and 24 h after liposomes injection (500 nmol total lipids/mouse). After centrifugation (500 g for 10 min) and filtration (0.22 μm cellulose acetate filters from Millipore, France), 100 μL of mouse serum were analyzed by size exclusion chromatography column (Superdex® 200) to check the integrity of the liposomes by comparison of the chromatographic profiles of the two radiotracers. Fifty fractions of 1 mL were collected and counted with ^{125}I and ^{111}In windows, in a gamma counter.

3. Results

3.1. Encapsulation and surface radiolabeling

Under optimal conditions (Mougin-Degraef et al., 2006), high percentages of encapsulation were obtained (64 ± 2 mean ± S.D., $N = 5$) corresponding to 43 ± 12 nmol BH encapsulated per μmol of total lipids, for an incubation time of 30 min at 37 °C. The surface radiolabeling efficiency was above 77 ± 11% (mean ± S.D., $N = 5$) after 30 min at 37 °C.

3.2. In vitro radiolabeling stability

Five different types of liposome, EPC/Chol, DMPC/-Chol/DMPE-DTPA, DMPC/Chol/DMPE-DTPA/DMPE-PEG-2000 and DSPC/Chol/DSPE-DTPA/DSPE-PEG2000, with 5 mol% or 8 mol% of PEG, were tested for *in vitro* radiolabeling stability in human serum.

The surface radiolabeling stability, with 1.5 mol% DMPE or DSPE-DTPA liposomes, was above 90% up to 6 days in buffer at 4 °C as in serum at 37 °C, irrespective of the lipid composition.

The influence of the lipid composition on the ^{125}I-BH reagent retention in liposome is presented in Fig. 1. EPC/Chol and DMPC/Chol/DMPE-DTPA showed relatively low stability, with respectively 30% and 50% of ^{125}I-BH remaining associated to the liposomes at 24 h. Incorporation of PEG into liposomes

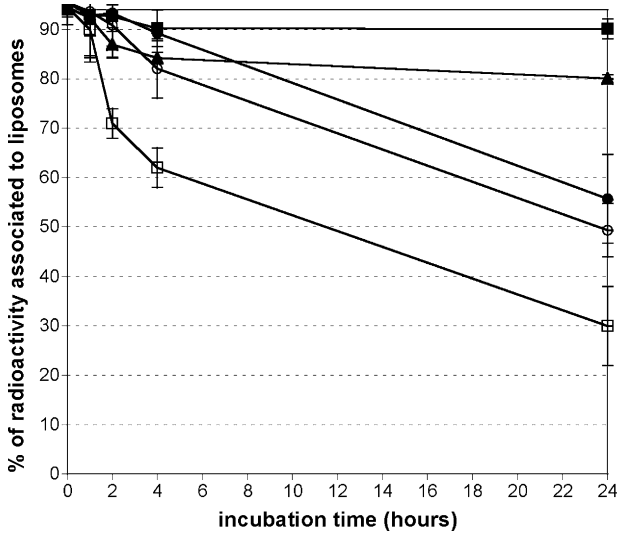

Fig. 1. Influence of lipid composition on liposomes radiolabeling stability *in vitro*. Liposomes were incubated in 100% human serum at 37 °C (μmol total lipids/mL serum). Each point represents the mean obtained from three experiments during 24 h and the error bars represent the standard deviation (S.D.) for ❑ EPC/Chol, ❍ DMPC/Chol/DMPE-DTPA, (●) DMPC/Chol/DMPE-DTPA/DMPE-PEG 5 mol%, (■) DSPC/Chol/DSPE-DTPA/DSPE-PEG 5 mol% and (▲) DSPC/Chol/DSPE-DTPA/DSPE-PEG 8 mol%.

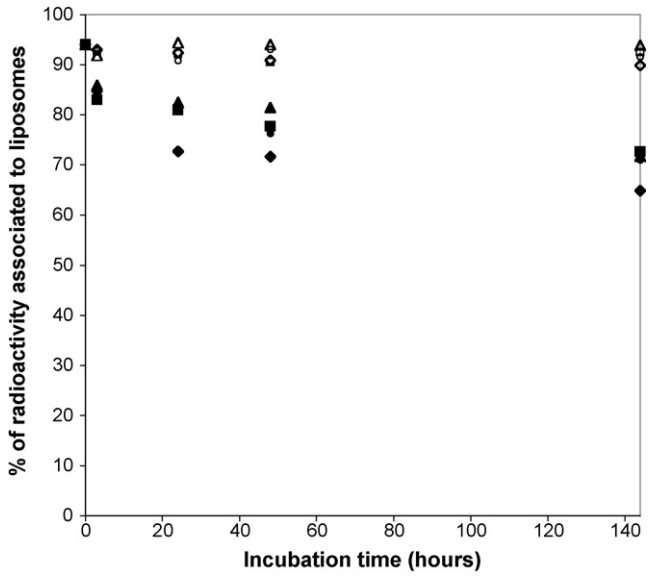

Fig. 2. Influence of lipid concentration on the ^{125}I-BH reagent retention *in vitro* in liposomes composed of DSPC/Chol/DSPE-DTPA/DSPE-PEG2000. The black symbols indicate the liposomes PEGylated with DSPE-PEG 8 mol% and the white symbols with DSPE-PEG2000 5 mol%. The symbols for the different concentrations are 2 μmol (●), 1 μmol (■), 0.1 μmol (▲) and 0.02 μmol (♦) total lipids per mL of 100% human serum at 37 °C.

composed of DMPC or DSPC increased the stability of ^{125}I-BH reagent encapsulation from 55% up to 80% at 24 h in human serum. Increasing DSPE-PEG2000 from 5 mol% to 8 mol% was found to reduce the stability after 2 h in serum. For the optimal formulation, DSPC/Chol/DSPE-DTPA/DSPE-PEG2000 with 5 mol% of PEG, the retention of ^{125}I-BH reagent in liposome in 4 °C buffer and in 37 °C serum was high, up to 90% after 6 days.

The influence of lipid concentration on radiolabeling stability was tested with PEGylated liposomes (Fig. 2). The data showed a slightly lower stability for the highest dilution (0.02 μmol total lipids/mL serum, compared to 0.1 μmol, 1 μmol or 2 μmol total lipids/mL serum). The low fraction of radioactivity (^{111}In as ^{125}I) released from the liposome demonstrates the high stability of the optimal formulation.

3.3. Pharmacokinetics and biodistribution

3.3.1. Pharmacokinetic parameters

The liposome formulations containing 5 mol% or 8 mol% of PE-PEG derivatives were selected for blood clearance evaluation over 24 h after injection in mice. As shown in Table 1, a rapid elimination of conventional liposome from blood circulation was observed (clearance = 1.44 ± 0.60 mL/h), suggesting that liposomes of this size range (100–200 nm) are readily taken by the reticuloendothelial system. Incorporation of DSPE-PEG 5 mol% or 8 mol% significantly increased the blood level of liposomes compared to conventional liposomes and showed almost identical half-life and clearance (clearance = 0.15 ± 0.01 mL/h and 0.18 ± 0.001 mL/h, respectively for DSPE-PEG 5 and 8%). The pharmacokinetic parameters were nearly the same with the two radiotracers (Table 1).

^{125}I-BH coupled to arginine was rapidly excreted from mice with a short half-life (clearance = 6.04 ± 1.50 mL/h). This rapid blood clearance of ^{125}I-BH-arginine was associated with very high levels of radioactivity excreted in the urine at early time-points (see Section 3.3.3).

3.3.2. Chromatographic profiles

Chromatography of serum samples on Superdex® 200 column are presented in Fig. 3a and b. The activity corresponding to radiolabeled liposomes was collected with a volume retention comprised between 9 mL and 14 mL (maximum at 11 mL). The sample taken 1 h, 5 h and 24 h after liposomes injection, showed the same chromatographic profiles with ^{111}In as well as with ^{125}I. After destruction with Triton 10× (100 μL, 1 h), the activity corresponding to free ^{125}I was collected between 39 mL and 43 mL (maximum at 40 mL) and phospholipids radiolabeled with ^{111}In were collected with a maximum at 20 mL. These data suggest that radiolabeling of liposomes is very stable in serum and the correlation between the two radiotracers (^{111}In for the surface and ^{125}I for the aqueous phase) confirms the integrity of the vesicles *in vivo* 24 h after injection.

3.3.3. Biodistribution and routes of elimination

The activity in blood and the uptake in the major organs of elimination (liver, spleen, kidneys, lung) are represented at 10 min, 1 h and 4 h for conventional liposomes (Fig. 4) and at 3 h, 24 h and 48 h for PEGylated, long-circulating liposomes (Fig. 5). The apparent difference in blood levels at 10 min for conventional liposomes was not significant and not confirmed in other experiments (not shown).

Blood contents at different times are the same for ^{125}I and ^{111}In (except at 10 min for conventional liposomes).

Table 1
Pharmacokinetic parameters

Formulation	Radionuclide	T1/2 α (h)	T1/2 β (h)	Clearance (mL/h)	Vd (mL)
DSPC/chol/DSPE-DTPA (67:31,5:1,5)	I-125	1.35 (0.28)	–	1.44 (0.60)	2.67 (0.59)
	In-111	1.31 (0.12)	–	1.68(0.21)	3.19 (0.60)
DSPC/chol/DSPE-DTPA/DSPE-PEG2000 (63:30,5:1,5:5)	I-125	0.71 (0.95)	14.64 (0.47)	0.15 (0.01)	2.73 (0.70)
	In-111	0.38 (0.46)	13.76 (0.98)	0.16 (0.01)	2.7 (0.83)
DSPC/chol/DSPE-DTPA/DSPE-PEG2000 (60:30,5:1,5:8)	I-125	0.83 (0.07)	14.11 (0.01)	0.18 (0.001)	2.35 (0.04)
	In-111	0.59 (0.05)	13.02 (1.24)	0.20 (0.01)	2.56 (0.42)
^{125}I-BH-arginine	I-125	0.25 (0.07)	13.30 (5.66)	6.04 (1.50)	17.53 (4.28)

Half-lives in blood ($T_{1/2}$), total body clearance (mL/h) and volume of distribution (Vd) for different liposomes formulations as estimated from iodine-125 and indium-111 data. Mice were injected i.v. with doubly radiolabeled conventional or PEGylated liposomes (500 nmol total lipids/mouse) and with ^{125}I-BH-arginine as a control.

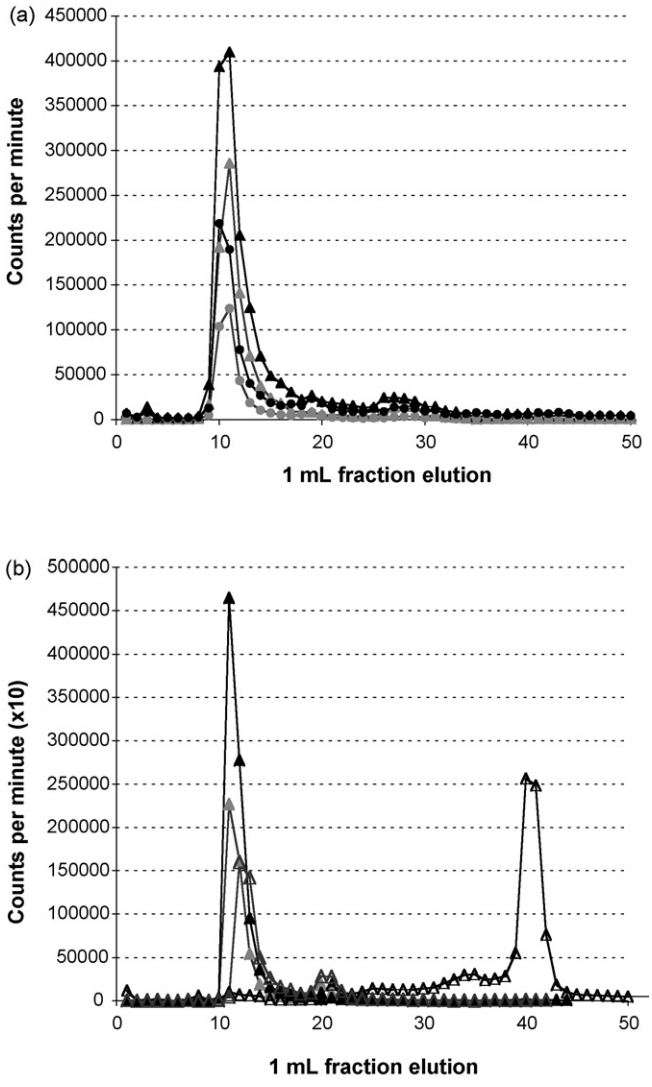

Fig. 3. Chromatographic profiles of mice serum. Blood samples were taken, centrifuged (500 g, 10 min) and filtered through a 0.22 μm filter and 100 μL of serum were injected on Sephadex® 200 column. Eluted fractions (1 mL) were collected and counted in ^{125}I and ^{111}In in a gamma counter. Panel A: serum collected 1 h (▲) and 5 h (●) (black for ^{125}I and grey for ^{111}In) after injection of doubly radiolabeled liposomes (DSPC/Chol/DSPE-DTPA/DSPE-PEG 5%, 500 nmol total lipids/mouse). Panel B: serum collected 24 h after liposomes injection, before and after 1 h incubation with Triton X100: radiolabeled liposomes (▲) and radiolabeled liposomes +Triton X100 (△).

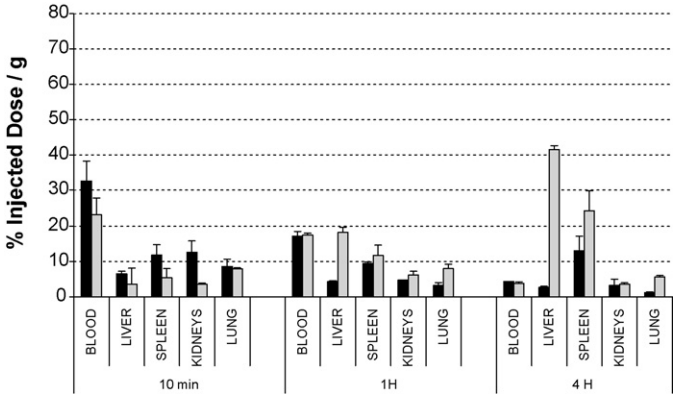

Fig. 4. Biodistribution in mice of doubly radiolabeled conventional liposomes. Mice were sacrificed and dissected 10 min, 1 h and 24 h after injection of DSPC/Chol/DSPE-DTPA liposomes (500 nmol total lipids/mouse). The data are expressed as the percentage of total administered liposome dose per gram of tissue (% ID/g, mean ± S.D., $n = 4$), in ^{125}I (in black) and ^{111}In (in grey).

The biodistribution study, performed with the best liposome formulation (DSPC/Chol/DSPE-DTPA/DSPE-PEG2000, 63:30.5:1,5:5), showed that more than 10% of the injected liposome dose remains in one gram of blood, 24 h after injection (14.2 ± 1.0 for ^{125}I and 13.5 ± 1.7% ID/g for ^{111}In), in contrast with conventional liposomes (4.3 ± 0.1 for ^{125}I and 3.8 ± 0.5% ID/g for ^{111}In, at 4 h),

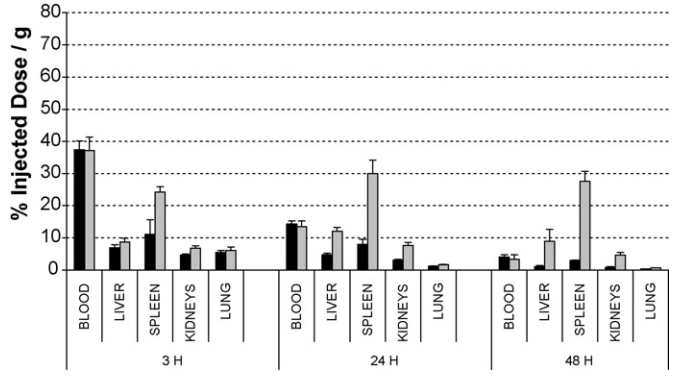

Fig. 5. Biodistribution in mice of doubly radiolabeled PEGylated liposomes with the optimal formulation. Mice were sacrificed and dissected 3 h, 24 h and 48 h after injection of DSPC/Chol/DSPE-DTPA/DSPE-PEG 5% liposomes (500 nmol total lipids/mouse). The data are expressed as the percentage of total administered liposome dose per gram of tissue (% ID/g, mean ± S.D., $n = 4$), in ^{125}I (in black) and ^{111}In (in grey).

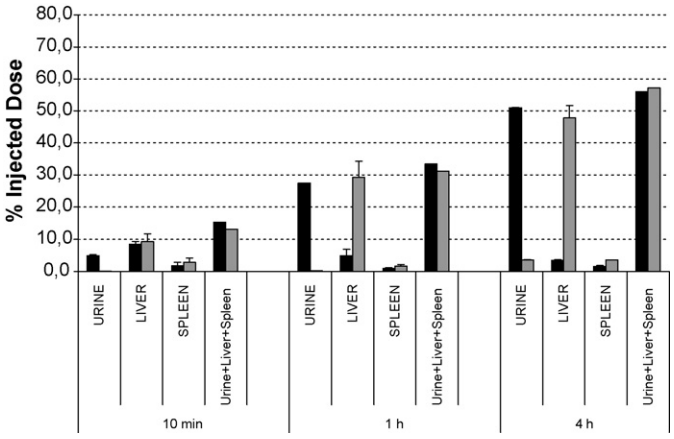

Fig. 6. Elimination of ^{125}I-BH-Arginine (in black) and ^{111}In-phospholipids (in grey) after injection of conventional liposomes (DSPC/Chol/DSPE-DTPA, 500 nmol total lipids/mouse). The data are expressed as the percentage of total administered liposome dose (% ID, mean ± S.D., $n = 4$), in urine, in major elimination organs (liver and spleen) and for the total eliminated fraction dose (urine + liver + spleen).

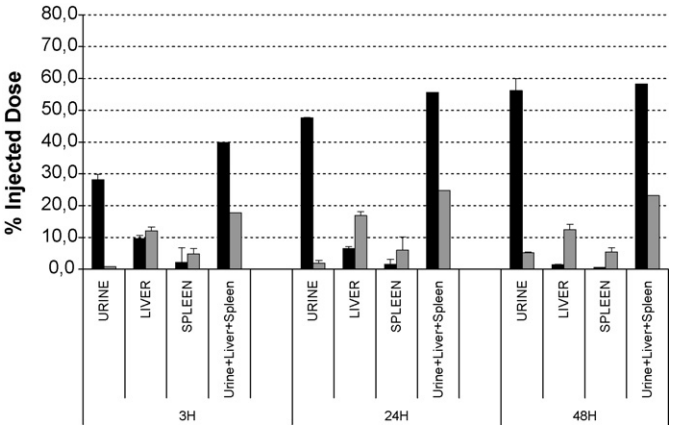

Fig. 7. Elimination of ^{125}I-BH-Arginine (in black) and ^{111}In-phospholipids (in grey) after injection of PEGylated liposomes (DSPC/Chol/DSPE-DTPA/DSPE-PEG 5 mol%, 500 nmol total lipids/mouse). The data are expressed as the percentage of total administered liposome dose (% ID, mean ± S.D., $n = 4$), in urine and in major elimination organs (liver and spleen) and for the total eliminated fraction dose (urine + liver + spleen).

After destruction of the liposomes by the reticuloendothelial system, it is expected that the routes of elimination be different for ^{111}In coupled to phospholipids and for ^{125}I-BH coupled to arginine. So we investigated the eliminated fraction dose in urine, liver and spleen, and the total activity accumulated with these three major routes of elimination. With conventional liposomes (Fig. 6), the high levels of ^{125}I found in urine 4 h after injection (51.0 ± 0.1% ID) is explained by the rapid renal excretion of ^{125}I-BH coupled to arginine after liposome disruption. In the same time, the relatively low levels of ^{111}In in urine (3.6 ± 0.2% ID) and the accumulation in liver and spleen (respectively, 47.8 ± 3.9% ID and 3.6 ± 0.1% ID, at 4 h) confirm the uptake of radiolabeled phospholipids released from degraded liposomes. At each time, the total elimination showed the same activity for ^{125}I and for ^{111}In. PEGylated liposomes accumulated also in liver and spleen but at a slower rate and activity uptake was significantly lower (respectively, 16.9 ± 1.2% ID and 6.0 ± 1.6% ID, in ^{111}In at 24 h) as shown in Fig. 7 Again ^{125}I-BH coupled to arginine was mostly eliminated in the urine. Indium uptake in liver and spleen was slower with the long circulating liposomes and indium may be excreted in the feces, which were not monitored in this study. This may explain the lower activity measured at any one time in these organs. Other routes of elimination may not be excluded but this study does not make it possible to really conclude about other ways of liposome catabolism.

4. Discussion

To be useful for targeted radionuclide therapy, liposomes must carry high loads of radioactivity and radiolabeling must be performed just prior to use to reduce radiolysis. We have thus developed a method, based on the active-loading approach, that uses a chemical reaction to transform a neutral radioiodinated molecule capable of crossing liposomes membranes into a charged, water soluble and membrane impermeable compound, which remains entrapped in the liposome aqueous core. Radio-iodinated BH reagent can cross the liposome membrane and react inside the liposome with encapsulated arginine. The reaction is complete within 30 min and the encapsulation yield is more than 60%. The maximum loading correspond to approximately 56 nmol of BH per μmol of lipids for liposome containing 80 mM arginine and 100% of the entrapped activity correspond to the arginine-BH adduct. Considering that 50 nmol BH may be encapsulated per μmol of lipid and a specific activity of 55.5 MBq (1.5 mCi) per nmol of BH, which can be routinely achieved (data not shown), 2.8 GBq (75 mCi) of iodine-131 could be encapsulated per μmol of lipids. This is much higher than with any other method published previously.

For therapeutic applications, it is necessary that the radiolabeling be stable *in vitro* and *in vivo*. The aim of this study was to optimize the liposome formulation in order to obtain favorable pharmacokinetics parameters and high radiolabeling stabilities after intravenous injection to mice. By radiolabeling both the liposome membrane and the liposome inner aqueous core, the liposome integrity could be checked after *in vivo* injection. The fate of the two tracers was monitored during all studies.

In vitro, the radiolabeling of the membrane was stable (>90%) irrespective of the liposome formulation, which is easily understood since ^{111}In complexation with DTPA is very stable and the phospholipid derivative is directly inserted in the liposome membrane. Stability of the ^{125}I-BH-arginine adduct encapsulation upon incubation in serum could be more problematic as serum constituent may make the liposome membrane leaky to small molecular weight solutes. Results showed that the stability in buffer at 4 °C was quite satisfactory, with less than 10% leakage after 6 days, but the influence of the formulation was preponderant on the *in vitro* stability of aqueous phase radiolabeling. As shown in Fig. 1, with fluid liposome compositions, such as EPC/Chol or DMPC/Chol, 50% to 70% of the activity leaked out of the liposomes within 24 h in pure human serum at 37 °C. Thereafter leakage slowed down. For rigid liposomes

composed of DSPC/chol and DSPE-PEG2000, activity leakage was less than 15% in 6 days in pure human serum at 37 °C. Lipid concentration had little influence on radiolabeling stability between 0.02 μmol and 2 μmol of total lipids per mL of human serum at 37 °C. The small stability difference observed between liposomes with 5% or 8% PE-PEG has already been reported in literature (Maruyama and al., 1992) and was confirmed here *in vivo* with the clearance of these two formulations, respectively 0.15 ± 0.01 mL/h and 0.18 ± 0.001 mL/h (Table 1). The pharmacokinetic parameters after intravenous injection in mice were also dependent on the liposome formulation. As expected, conventional liposomes (DSPC/Chol/DSPE-DTPA) had a fairly fast clearance of 1.44 ± 0.60 mL/h. PEG increased by a factor of 9 the residence time in blood of rigid liposomes (DSPC/Chol/DSPE-DTA/DSPE-PEG) but not so much of fluid liposomes, that confirms the literature results (Maruyama et al., 1992; Senior et al., 1991). The good correlation between the two tracers in all cases indicated that we were monitoring the pharmacokinetics of intact liposomes. Radiochromatography of mouse serum by HPLC confirmed the integrity of the liposomes.

Then, biodistribution data should reflect the distribution of intact liposomes. In this work, liposomes with 5% PE-PEG, which showed the longest blood residence time, were compared with conventional liposomes (respectively Figs. 5–7 and 4–6). Whereas blood levels evaluated with the two tracers (membrane and aqueous phase) were the same at different times after injection, the indium-labeled phospholipids remained in liver and spleen. Prominent uptake of ^{111}In in liver and spleen demonstrates the accumulation of radiolabeled lipids in liposome catabolizing organs, whereas the radioiodinated BH-arginine adduct was quickly eliminated from blood after liposome disruption. Indeed, non-encapsulated BH-arginine adduct is quickly eliminated in urine (clearance 6.04 mL/h). The activity in urine (% ID) reflected the iodine elimination whereas only relatively little indium activity was collected in urines. The total activity eliminated (urine + liver + spleen) was the same for conventional liposomes, but with long-circulating liposomes the difference between the two radiotracers increased over time, which suggest a different route of metabolism for ^{111}In coupled to phospholipids.

In conclusion, in the present study we have investigated different liposome formulations radiolabeled both in their membrane and inner aqueous phase. Liposomes composed of DSPC/Chol/DSPE-DTPA and 5 mol% DSPE-PEG2000 were found to be stable and to have a long blood residence time. Extemporaneous aqueous phase radiolabeling by active loading of radioiodinated BH in preformed liposomes allows the liposomes to carry high radionuclide activities. Attempts at pretargeting these liposomes to tumors by means of bispecific antibodies are in progress. The significant reduction of activity uptake in organs that catabolize the liposomes (liver and spleen), achieved by using hydrophilic activity carriers loaded in the liposome aqueous phase, and the rapid renal elimination of released radioactivity should be a considerable advantage for therapeutic applications by reducing the irradiation dose delivered to normal tissues.

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