Novel Long-Circulating Lipid Nanocapsules

Didier Hoarau,[1,2] Pascal Delmas,[2] Stéphanie David,[1] Emmanuelle Roux,[1] and Jean-Christophe Leroux[1,3]

Received March 2, 2004; accepted June 21, 2004

Purpose. To develop and evaluate novel long-circulating lipid nanocapsules (LN) designed for tumor delivery of lipophilic drugs.
Methods. Nanocapsules were produced by a solvent-free phase inversion process and were coated with polyethylene glycol-distearoylphosphatidylethanolamine conjugate (DSPE-PEG) during preparation or by a post-insertion step. *In vivo* studies were conducted in rats to assess LN pharmacokinetics and biodistribution.
Results. Post-insertion of DSPE-PEG appeared to be a convenient and effective method of obtaining LN of controlled sizes with high PEG density at their surface. After intravenous injection to rats, PEGylated lipid nanocapsules obtained by the post-insertion method exhibited long-circulating properties. Up to 50% of the injected dose was still present in the blood 8 h after administration for LN containing 6 mol% PEG 5000 or 10 mol% PEG 2000. This represented an area under the blood concentration-time curve of almost 70% that of liposomes used in the Doxil® formulation.
Conclusion. With a simple solvent free-process, it was possible to produce long-circulating LN of controlled sizes. Such LN could prove useful for the passive delivery of lipophilic anticancer drugs to solid tumors.

KEY WORDS: lipid nanocapsules; long-circulating colloids; injectable drug carrier; drug delivery systems; poly(ethylene glycol); biodistribution; pharmacokinetics.

INTRODUCTION

Colloidal drug carriers, such as liposomes (1), nanoparticles (2), and polymeric micelles (3), have been investigated for drug delivery applications for more than three decades. Perhaps the most exciting achievements with submicronic systems have been made in the fields of cancer chemotherapy and diagnosis. It is now well established that passive targeting of solid neoplasms by systemically administered drug carriers requires both long-circulating properties and adequate particle size for optimal extravasation at tumoral sites (1). Particles that are too small (<50 nm) are either readily eliminated by renal filtration or lost by nonspecific extravasation at the liver or bone marrow sinus endothelia. On the other hand, large nanoparticles (>200 nm) are rapidly cleared by splenic filtration (4,5). Colloids in the range of 50–200 nm have been shown to accumulate in solid tumors to a significant extent and improve the therapeutic index of several anticancer drugs.

Long-circulating colloids are generally obtained by grafting polyethylene glycol (PEG) derivatives at the carrier surface. For instance, the insertion of polyethylene glycol-distearoylphosphatidylethanolamine conjugates (DSPE-PEG) into phospholipid bilayers provides liposomes and their encapsulated cargo with a long half-life ($t_{1/2}$ > 20 h) after intravenous (iv) injection. In humans, it was found that the area under the plasma concentration-time curve (AUC) of doxorubicin entrapped into PEGylated liposomes increased at least 10-fold over conventional liposomes (1). Owing to its hydrophilicity and chain flexibility, PEG creates a zone of steric hindrance that slows down liposomal clearance by the mononuclear phagocyte system (MPS), possibly by decreasing the rate and extent of opsonin binding to liposomes (6) and/or by interfering with the association of liposome-bound opsonins with macrophage receptors (7). Both the range and magnitude of the steric effect increase with greater PEG surface density and molecular weight (M_w). These surface-modified vesicles, also referred to as Stealth® liposomes, show enhanced accumulation at tumor sites due to the "enhanced permeation and retention" (EPR) effect, which is characterized by increased vascular permeability and decreased lymphatic drainage at tumoral sites (8). One of the main limitations of liposomes is their poor loading capacity toward hydrophobic drugs that cannot be dissolved in large amounts in the phospholipid bilayer or sequestered in the liposomal aqueous core. Furthermore, after iv administration, such drugs are often rapidly extracted from the bilayers and distribute to other hydrophobic sites in the body, precluding any specific accumulation at the target sites (9). Hence, oil-based colloidal formulations (10) and polymeric nanocarriers (2,3) appear to be attractive alternatives for water-insoluble drugs, given that they can be dissolved within the oily core or hydrophobic matrix of these systems with high entrapment efficiencies.

Recently, lipid nanocapsules (LN) with a core-shell structure were proposed by the group of Benoit as novel carriers for lipophilic drugs (11). The structure of these core-shell particulates was found to be a hybrid between polymeric nanocapsules and liposomes. These capsules are composed of FDA-approved medium chain triglycerides (TG) (core) and a combination of hydrophilic/lipophilic surfactants (shell). Heurtault *et al.* (11,12) demonstrated that mean size and size distribution of LN can be precisely controlled and that the semi-rigid shell imparts remarkable physical stability to the formulations. Such nanocapsules can accommodate relatively high concentrations of lipid-soluble drugs in their oily core. Unlike liposomes and most nanoparticle formulations, these nanostructures, which present a monodisperse size distribution, are prepared *via* a solvent-free process that does not require any purification step. Despite these appealing characteristics, plain LN exhibit an early half disappearance time ($T_{50\%}$) of 45 min after iv injection in rats (13), which is too short to allow accumulation in most solid tumors. As PEG is known to decrease the scavenging rate of a number of colloids, including polymeric nanoparticles (14), we investigated, in this study, whether the insertion of DSPE-PEG in the LN shell could improve LN circulation time without compromising the unique attributes of the formulation and preparation procedure. Two different DSPE-PEG incorporation methods were evaluated, and the pharmacokinetics and biodistribution

[1] Canada Research Chair in Drug Delivery, Faculty of Pharmacy, University of Montreal, C.P. 6128 Succ. Centre-ville, Montreal (Qc) Canada H3C 3J7.
[2] Ethypharm Inc., 200 bd Armand Frappier, Laval (Qc), Canada H7V 4A6.
[3] To whom correspondence should be addressed. (email: Jean-Christophe.Leroux@umontreal.ca)

of the PEGylated LN formulations were assessed after systemic administration to healthy rats.

MATERIALS AND METHODS

Materials

Labrafac® CC (caprylic/capric TG) and the non-ionic hydrophilic surfactant Solutol® HS15 (PEG 660 12-hydroxystearate) were kind gifts from Gattefossé S.A. (Saint-Priest, France) and BASF (Ludwigshafen, Germany), respectively. Lipoid® S75-3 (hydrogenated soybean lecithin with 70% phosphatidylcholine) was generously provided by Lipoid® GmbH (Ludwigshafen, Germany). Hydrogenated soybean phosphatidylcholine (HSPC, >99% phosphatidylcholine) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-monomethoxy-[PEG 2000] (DSPE-PEG_{2000}) were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). DSPE-PEG_{5000} and cholesterol were procured from Avanti Polar Lipids Inc. (Alabaster, AL, USA). [^{3}H]-cholesteryl hexadecyl ether (CHE) was purchased from Perkin Elmer (Woodbridge, ON, Canada). All products were used without further purification. Water was deionized with a MilliQ® purification system (Millipore, Bedford, MA, USA) before use.

Preparation of Conventional Nanocapsules

Plain LN were prepared by a phase inversion-based process, as described previously (11). Briefly, the constituents of the shell (Solutol® HS15 and Lipoid® S75-3 or HSPC), of the core (Labrafac® CC) and of the dispersing phase (~4.4% w/v NaCl in water) were combined in different proportions for a total mass of 5 g. Typically, the lipid phase represented 17–22% of total mass. The different components were mixed under magnetic stirring and heated above the phase transition temperature (ca. 70°C). The mixture was cooled down to 60°C and the o/w nanoemulsion obtained was again heated above the phase inversion zone to give a *w/o* system. This cycle was repeated twice, and the o/w emulsion was quenched by the addition of 12.5 ml water at 2°C. The LN suspension was stirred for 10 min before use.

Preparation of PEGylated Nanocapsules

PEGylated nanocapsules were obtained by two different procedures referred to as the conventional and post-insertion methods. With the conventional method, DSPE-PEG was added in the initial mixture of lipids and water, and nanocapsules were produced as described above. Nanocapsules with higher DSPE-PEG incorporation ratios were prepared by the post-insertion procedure as reported elsewhere for liposomes (15). Briefly, plain LN were incubated for 90 min with an aqueous micellar solution of DSPE-PEG at 60°C. The suspension was vortexed every 15 min and then quenched in an ice bath for 1 min. The final DSPE-PEG concentration corresponded to 6 or 10 mol% of total surface lipids (i.e., excluding TG).

Preparation of Radiolabeled Nanocapsules

[^{3}H]-radiolabeled LN were prepared as follows. The lipids and surfactant were first dissolved in chloroform and mixed with CHE. This cholesterol derivative was chosen as radiolabeled tracer because it is a non-exchangeable lipid (16). The solution was dried at 60°C under an argon stream. Complete solvent evaporation was achieved by placing the lipids *in vacuo* (0.1 mbar) for at least 30 min. A saline solution (NaCl 5.8% w/v) was added to the lipid mix, and the preparation was subjected to the temperature cycles described above. DSPE-PEG was incorporated by the conventional or post-insertion method. The formulations were subsequently diluted with NaCl 0.9% (w/v) to adjust the lipid dose for injection.

Preparation of Radiolabeled, Long-Circulating Liposomes

Control unilamellar, long-circulating liposomes with the same lipid composition as Doxil® (without the drug), that is, HSPC/Chol/DSPE-PEG_{2000} (56:39:5 molar ratio), were prepared by the thin film hydration method. Briefly, lipids dissolved in chloroform were mixed with a trace amount of CHE. After solvent evaporation, the dried lipid film was placed *in vacuo* (0.1 mbar) for at least 30 min to remove residual solvent. The film was then hydrated with NaCl 0.9% solution. The liposomes were finally extruded several times at 60°C through 100- and 50-nm polycarbonate filters using a LiposoFast extruder (Avestin, Ottawa, ON, Canada) to yield ca. 100-nm vesicles. Lipid loss, evaluated by radioactive counts before and after extrusion, was 0.4%.

Size Measurements

Mean hydrodynamic diameter (peak) and size distribution (width) of LN and liposomes were assessed at 25°C by dynamic light scattering with a Coulter N4Plus (Coulter Electronics, Miami, FL, USA) at a fixed angle of 90°. Measurements were performed in triplicate after dilution of the suspension in water.

Atomic Force Microscopy (AFM) Imaging

The topographic data of the LN in suspension was collected using a Nanoscope III Dimension 3100 atomic force microscope (Digital Instruments, Santa Barbara, CA, USA). Imaging was performed in tapping mode using a silicon tip operating at a 300-kHz resonant frequency and a 50 N/m constant force. Samples were prepared by depositing a suspension of diluted LN on mica surface at room temperature. The system was allowed to equilibrate for 1 h.

In Vivo Studies

Animal care and studies were approved by the Animal Welfare and Ethics Committee of the University of Montreal. Pharmacokinetics and tissue distribution of the radiolabeled formulations were investigated in male Sprague-Dawley rats weighing 300–350 g (Charles River, St-Constant, QC, Canada). The animals (n = 3 to 5 per group) were surgically prepared for iv injections and arterial blood sampling according to the procedure of Moreau *et al.* (17). Briefly, polyethylene catheters, externalized at the back of the neck and protected with a tethering system, were inserted into the femoral vein and artery, and the rats were allowed to recover for at least 24 h with water and food *ad libitum*. The catheters were filled with physiologic saline and heparin solution to prevent

clot formation. Four hundred microliters of radiolabeled formulations (2 mg of lipids, 3 μCi/rat) were injected *via* the vein cannula. The time points for blood collection (0.4 ml *via* the arterial cannula) were 5, 15, 30 min and 1, 2, 4, 8, 12, and 24 h after injection. Animals were sacrificed just after the last blood sampling point and exsanguinated with a physiologic solution prior to organ removal (spleen, liver, lungs, kidney, heart). Organ samples were introduced into scintillation vials and dissolved in Soluene® 350 tissue solubilizer (Perkin Elmer). The vials were placed in an oven at 60°C until total tissue solubilization occurred. Accurately-weighed blood samples were mixed with Soluene® 350 and isopropanol, digested for 1 h at 60°C prior to bleaching with 30% hydrogen peroxide. Ten milliliters of Hionic Fluor® scintillation cocktail (Perkin Elmer) were added to organ and blood samples. The vials were left to stand in the dark overnight at 4°C, and radioactivity was measured in a scintillation counter (Liquid Scintillation Analyser Tri-Carb 2100TR, Packard, Meriden, CT, USA). Blood concentrations of LN and liposomes at the various time points were calculated on the assumption that blood represents 7.5% of rat body weight (18).

Data Analysis

Pharmacokinetic data were treated by noncompartmental analysis of percentage-of-injected dose vs. time profiles. Several parameters were studied to compare the different formulations. The time corresponding to removal of 50% of the injected dose ($T_{50\%}$) was calculated by linear interpolation. The trapezoidal rule was used to calculate the AUC during the whole experimental period ($AUC_{[0\text{-}24]}$), the first 8 h ($AUC_{[0\text{-}8]}$), as well as the area under the first moment curve (AUMC) (Eq. 1). Extrapolation of the AUC and AUMC to infinite time could not be calculated due to the residual radioactivity remaining in the blood, which yielded non-reliable terminal elimination constants. Thus, mean residence time (MRT) was calculated from Eq. 2 up to t = 8 h.

$$AUMC_{0\to n} = \int_0^n t \cdot C \cdot dt = \sum_0^n \left(\frac{t_i \cdot C_i + t_{i+1} \cdot C_{i+1}}{2} \right) \cdot (t_{i+1} - t_i) \quad (1)$$

$$MRT_{0\to 8} = \frac{AUMC_{0\to 8}}{AUC_{0\to 8}} \quad (2)$$

All data above were expressed as mean ± SD. Statistics were computed with the JMP software version 5.0.1 (SAS Institute Inc., Cary, NC, USA). Differences in group means (multiple comparisons) were calculated by standard analysis of variance followed by the Tukey-Kramer test to determine the significance of all paired combinations. The homogeneity of variances across groups was first verified by Bartlett's test. A p value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Preparation of PEGylated Nanocapsules

PEGylated LN were first prepared by emulsifying the oil phase in the presence of DSPE-PEG (conventional method). Figure 1 shows the effect of the surfactant-to-triglyceride ratio (i.e., S/TG ratio) on mean nanocapsule diameter using two different concentrations of $DSPE\text{-}PEG_{2000}$. It can be seen

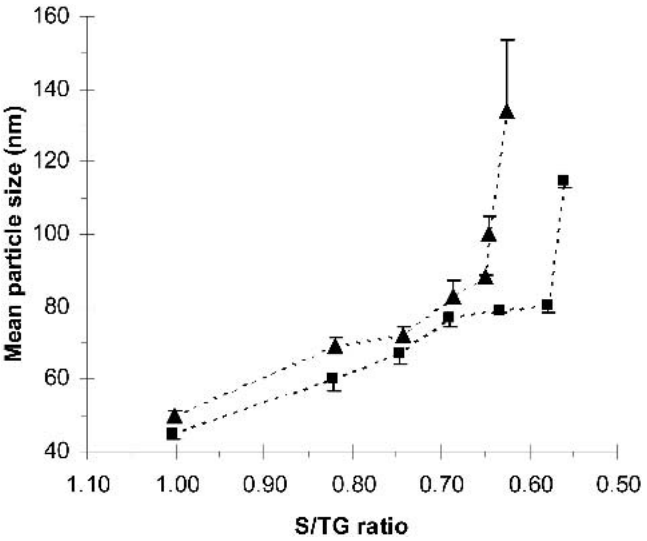

Fig. 1. Mean diameter (peak) of LN (Solutol®/Labrafac®/Lipoid® S75-3) prepared by the conventional method as a function of S/TG ratio. The $DSPE\text{-}PEG_{2000}$ proportions were fixed at 16 (squares) and 22% w/w (triangles) relative to the amount of phospholipid. (mean ± SD, n = 3). At 22% (w/w), the DSPE-PEG concentration ranged from 1.21 (50 nm) to 1.72 mol% (134 nm). At 16% (w/w), it varied between 0.82 (45 nm) and 1.41 mol% (115 nm).

that LN mean size increased almost linearly from 40 to 50 nm to 80 nm when the S/TG ratio decreased from 1 to 0.6–0.75, respectively. The LN formulations obtained within this S/TG range were stable for at least 3 months at room temperature in terms of particle size and size distribution (data not shown). Below this ratio, that is, at low hydrophilic surfactant concentrations, diameter rose sharply, and phase separation generally occurred within a few hours or days. Such a behavior was previously reported for LN prepared in the absence of DSPE-PEG (11). Heurtault *et al.* (11) indeed demonstrated that the feasibility domain where monodisperse and stable LN (20–100 nm) could be produced was strongly dependent on the proportions of hydrophilic surfactant, oil (TG) and water in the feed. These authors noted that phase separation occurred at low S/TG ratios. A similar trend, although less pronounced, was also observed when Lipoid® (phospholipid of the LN shell) was substituted by HSPC (data not shown).

Similarly, at predetermined S/TG ratios, LN size increased with DSPE-PEG content (Fig. 2). Unstable formulations were obtained when the $DSPE\text{-}PEG_{2000}$ and $DSPE\text{-}PEG_{5000}$ molar concentration *vs* total surface constituents exceeded 3.4 and 1.5 mol%, respectively. Thus, DSPE-PEG interfered with the emulsification or phase inversion process possibly because of its amphiphilic nature. Indeed, DSPE-PEG micellizes in the micromolar range (19), a concentration range which is far below that used for the LN preparation. Therefore, excess DSPE-PEG that does not insert at the lipid/water interface forms micelles that can extract surface components of the emulsified phase, and modify the interfacial properties of the system (11). Thus, with the conventional method, only relatively low amounts of PEG could be inserted in the LN shell.

Accordingly, it was decided to anchor the PEG-lipid derivative on pre-formed nanocapsules (post-insertion method). The post-insertion technique has been used for PEGylation

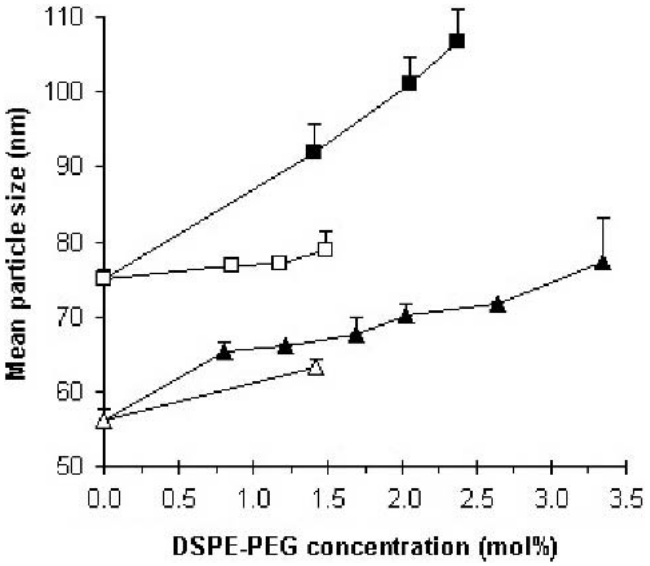

Fig. 2. Mean diameter (peak) of LN (Solutol®/Labrafac®/HSPC) obtained by the conventional method as a function of DSPE-PEG_{2000} (closed symbols) or DSPE-PEG_{5000} (open symbols) concentration relative to total surface components. The S/TG ratio was fixed at 0.82 (squares) or 1.0 (triangles) (mean ± SD, n = 3).

or incorporation of targeting peptide moieties into the liposomal outer bilayer (20–22). Post-insertion in HSPC-containing liposomes was shown to occur at temperatures below the phase transition of HSPC (T_c 58°C), the process being, however, kinetically longer and incomplete than post-insertion at 60 or 70°C. In the case of liposomes, Uster *et al.* (15) demonstrated that a plateau was reached after 60-min incubation at 60°C. Hence, to ensure complete insertion, DSPE-PEG was incubated with the LN for 90 min at 60°C. In this case, DSPE-PEG did not interfere with the emulsification or phase inversion processes, and size could be precisely adjusted within a wider range by simply varying the S/TG ratio (11). In addition, much higher proportions of DSPE-PEG (6-10 mol%) could be added to the formulation, irrespective of PEG chain length. Figure 3 shows that post-insertion of 6 mol% DSPE-PEG_{2000} resulted in a 5- to 8-nm increase in nanocapsule diameter. This size increment corresponds approximately to the thickness of a PEG coating (M_w = 2000) in brush conformation (15). Similarly, increasing PEG M_w 2000 to 5000 seemed to slightly augment mean LN diameter (not statistically significant). AFM analysis of the samples in the liquid state revealed spherical nanocapsules with a narrow size distribution (Fig. 4), as previously described for uncoated LN (11). The PEGylated LN obtained by the post-insertion method were physically stable for at least 12 months at room temperature in terms of mean diameter and size distribution (data not shown).

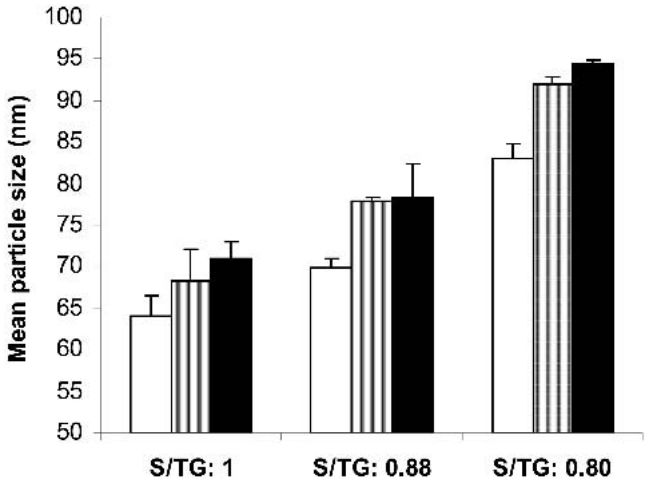

Fig. 3. Mean diameter (peak) of LN before (open columns) and after incorporation of 6 mol% DSPE-PEG_{2000} (shaded columns) or DSPE-PEG_{5000} (closed columns) at different S/TG ratios (mean ± SD, n = 3).

Pharmacokinetics and Biodistribution

The influence of PEG concentration and chain length on LN pharmacokinetics and biodistribution was investigated in rats after iv injection. The different formulations that were administered are listed in Table I. They all had a mean peak diameter between 60 and 95 nm and displayed a monodisperse size distribution. Each rat received a total of 2 mg lipids to avoid saturation of the MPS. Plain LN were rapidly cleared from blood (Fig. 5A, Table II), confirming the results of a previous pharmacokinetic study, which reported a systemic T_{50} of ~45 min in rats (13). In both investigations, less than 10% LN were still in the bloodstream 2 h after injection.

LN with 1.7 mol% DSPE-PEG_{2000} (2k-1.7c) only showed a 7% improvement in the AUC compared to plain LN, with most of the formulation cleared within 2 h. This improvement was, however, non-significant (Table II). Incorporation of 3.4 mol% of DSPE-PEG_{2000} (2k-3.4c) or 1.4 mol% DSPE-PEG_{5000} (5k-1.4c) *via* the conventional method significantly improved the circulation time, with 50–60% and ~20% of the dose still present in the blood 2 and 4 h after injection, respectively. These two formulation AUCs were ~3 times higher than that of uncoated LN (with a noticeable improvement in $T_{50\%}$) but corresponded only to 40% that of PEGylated liposomes (Table II). On a mol% basis, PEG_{5000} was found to be more efficient than PEG_{2000} in prolonging circu-

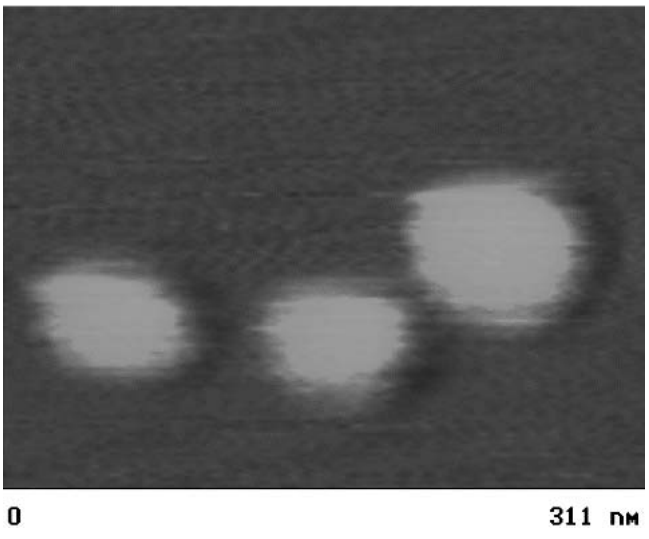

Fig. 4. AFM image of LN (Solutol®/Labrafac®/HSPC) coated with DSPE-PEG_{2000} (6 mol%). Imaging was performed in tapping mode in water. Nanocapsule diameters measured by cross-sections (data not shown) were from left to right: 56, 61, and 68 nm.

Table I. Characteristics of LN Formulations

Formulation	Phospholipid	PEG MW	PEG content (mol%)	Incorporation method[a]	Hydrodynamic diameters	
					Peak (nm)	Width (nm)
Liposomes	HSPC	2000	5.0	c	110	18
Plain LN	Lipoid®	—	0	—	56	23
2k-1.7c	Lipoid®	2000	1.7	c	75	12
2k-3.4c	HSPC	2000	3.4	c	76	12
5k-1.4c	HSPC	5000	1.4	c	61	15
2k-6i	HSPC	2000	6.0	i	84	12
5k-6i	HSPC	5000	6.0	i	93	12
2k-10i	HSPC	2000	10.0	i	82	11

[a] c = conventional; i = post-insertion.

lation time. This could be related to higher cloud density at the surface due to the increased number of possible PEG chain conformations as their size increases (23). As aforementioned, the effect of higher proportions of PEG inserted by the conventional method could not be tested because the resulting formulations were physically unstable.

Conversely, PEGylated LN obtained by the post-insertion method exhibited a dramatic change in their pharmacokinetic parameters (Fig. 5B, Table II). Sixty percent of the 6–10 mol% DSPE-PEG_{2000} (2k-6i and 2k-10i) and 6 mol% DSPE-PEG_{5000} (5k-6i) formulations were still circulating 4 h after injection. At t = 8 h, formulations 5k-6i and 2k-10i were present in the blood at ~50% of the injected dose. The AUC of these LN corresponded to 60–70% of that of control stealth liposomes (Table II). Raising the DSPE-PEG_{2000} concentration from 6 to 10 mol% resulted in significant 31% enhancement in the AUC (Table II) with an upward shift in $T_{50\%}$ from 5.1 to 7.8 h. At 6 mol% PEG, the mean AUC and $T_{50\%}$ values of the 5k-6i formulation were higher than those of 2k-6i, but not significantly different according to the multiple means comparison test. During the first 8 h after injection, the MRT of 5k-6i and 2k-10i LN were not statistically different from that of the control liposomal formulation (Tukey-Kramer test of $MRT_{[0-8]}$, data not shown). Surprisingly, a sharp decrease in blood concentrations occurred between 8 and 12 h after injection. Although this observation cannot be rationalized yet, one can hypothesize that PEG-lipid derivative progressively desorbed from the LN shell over time and then reached a threshold surface concentration, which was associated with rapid nanocapsule uptake by the MPS. Such a desorption may occur more rapidly for LN than liposomes because DSPE-PEG is expected to interact more favorably with the liposomal phospholipid bilayer than with TG having relatively short alkyl chains.

Nanocapsule uptake by the different organs was also assessed 24 h after administration. As anticipated, the nanocapsules mainly deposited in the liver and spleen. As for liposomes, formulations exhibiting the longest circulation time (post-insertion series) demonstrated less accumulation in MPS organs (24). In terms of particle concentration, the stealth liposomes accumulated more in the spleen than in the liver, whereas an opposite trend was observed for LN. In general, liposomes were taken up to a lower extent by the liver than LN: 1.4 vs. 2.4 to 4.2% injected dose per gram of liver (Figs. 6A and 6B). This can be attributed to the greater uptake of LN by liver macrophages and to their lower mean

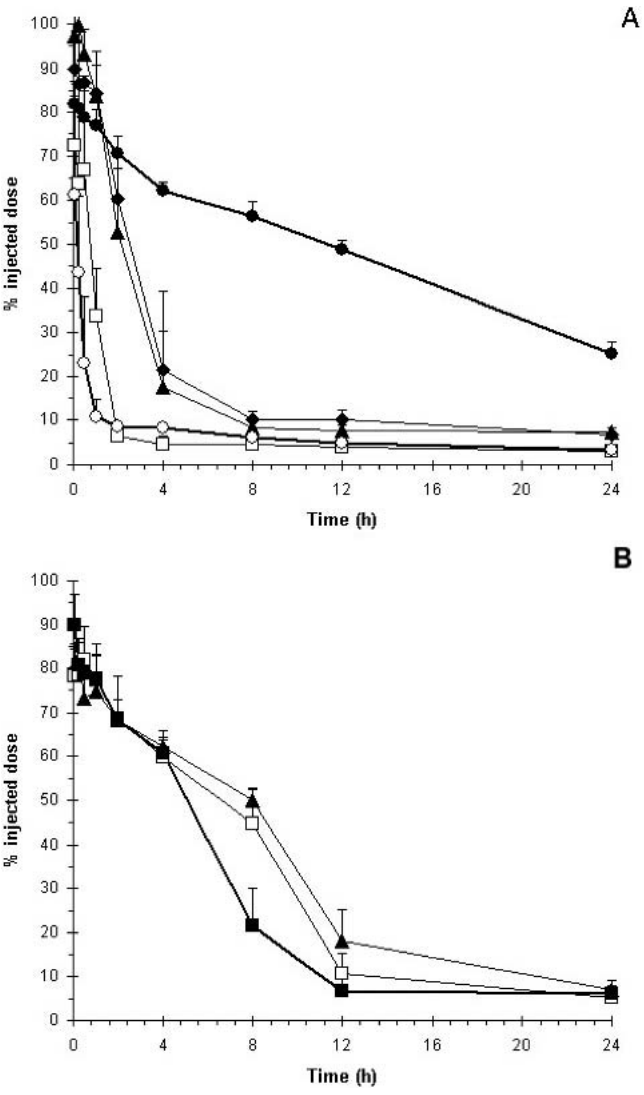

Fig. 5. Blood concentration-time profile of stealth liposomes and different LN formulations prepared by the conventional (A) or post-insertion method (B). Formulations were injected iv at a dose of 2 mg lipids/rat. Mean ± SD (n = 3 to 5). A: PEGylated liposomes (closed circles), plain LN (open circles), PEGylated LN 2k-1.7c (open squares), 5k-1.4c (closed triangles), and 2k-3.4c (closed diamonds). B: 2k-6i (closed squares), 5k-6i (open squares), and 2k-10i (closed triangles).

Table II. Pharmacokinetic Parameters of Injected Formulations

Formulation	n	$T_{50\%}$ (h)[a]	$MRT_{[0-8]}$ (h)[a]	$AUC_{[0-24]}$ (%dose · h)[a]	% AUC Liposomes	$AUC_{[0-24]}$ comparisons[b]
Liposomes	3	11.4 ± 0.87	4.2 ± 0.99	1181 ± 14	100	A
2k-10i	4	7.8 ± 0.68	3.6 ± 0.09	796 ± 63	67	B
5k-6i	5	6.4 ± 1.55	3.4 ± 0.13	682 ± 115	58	B C
2k-6i	4	5.1 ± 0.36	3.0 ± 0.20	607 ± 54	51	C D
2k-3.4c	4	2.7 ± 0.75	2.0 ± 0.49	481 ± 71	41	D E
5k-1.4c	4	2.2 ± 0.77	1.9 ± 0.29	418 ± 84	35	E
2k-1.7c	5	0.6 ± 0.31	1.8 ± 0.33	170 ± 44	14	F
Plain LN	3	0.2 ± 0.14	0.9 ± 0.52	159 ± 26	13	F

[a] Values are given as mean ± SD.
[b] Formulations not connected by the same letter are significantly different.

size *vs* liposomes, which may facilitate extravasation from the liver sinusoids (25). Liver uptake was less pronounced when the post-insertion method was used. Total LN accumulation in the liver and spleen amounted to 35% and 50% of the injected dose for 2k-10i and 2k-1.7c, respectively, *vs* 18% for stealth liposomes (data not shown). Surprisingly, after 24 h, LN seemed to accumulate to a higher extent than liposomes in the heart (Fig. 6). This could be explained by differences between the surface properties of the two carriers or by a different redistribution pattern of CHE.

Small size (30–150 nm) and long circulating properties are prerequisites for accumulation of colloidal carriers in solid tumors by the EPR effect. The post-insertion method allowed for the incorporation of higher amounts of DSPE-PEG compared to the conventional procedure. This resulted in substantial enhancement of LN circulation times. The AUC was indeed increased almost 5-fold when the PEG_{2000} concentration was raised from 1.7 to 10 mol% while the $T_{50\%}$ shifted from 0.6 to 7.8 h (Table II). Plain LN were previously claimed as being relatively long-circulating systems by Cahouet *et al.* (13). However, post-insertion of 10 mol% DSPE-PEG_{2000} produced a 5-fold increase in their AUC. Although plain LN bear a high density of short PEG segments at their surface (originating from Solutol®), these chains are too short (660 g/mol, 15 repeating ethylene oxide units) to allow sufficient protection against opsonization. The effectiveness of hydrophilic polymer chains in conferring stealth properties depends on both their length and density. Mori *et al.* (26) conducted a comparative study with liposomes containing surface-anchored PEG 750, 2000, and 5000 at an equal molar concentration. *In vitro*, PEG 750 proved ineffective in conferring steric protection against opsonization, whereas the longest PEG chains exhibited chain length-dependent efficiency. This *in vitro* behavior was correlated with *in vivo* half-lives after iv injection to mice, which were less than 30 min, approximately 2 h and more than 3 h for PEG 750, 2000 and 5000, respectively. Other studies (27) disclosed that below 1000 g/mol, PEG chains, even at surface concentrations approaching saturation, do not provide liposomes with adequate stealth properties.

The long-circulating LN presented in this work compare advantageously with other nanoparticulate systems reported in the literature. When biodegradable, long-circulating poly-(lactide-*co*-glycolide)-PEG nanospheres were injected iv to mice, between 60-70% and 70-90% of the total dose was cleared from the bloodstream 30 and 90 min after adminis-

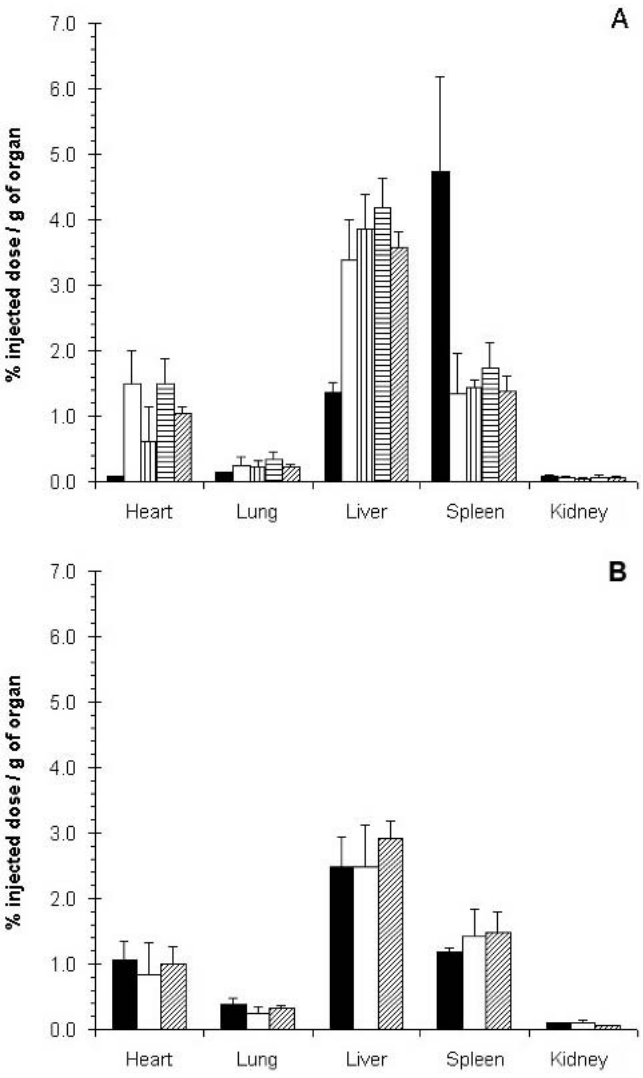

Fig. 6. Tissue distribution of stealth liposomes and different LN formulations prepared by the conventional (A) or post-insertion method (B). Radioactivity was assayed in the different organs 24 h after iv injection. Mean ± SD (n = 3 to 5). A: PEGylated liposomes (closed columns), plain LN (open columns), PEGylated LN 2k-1.7c (vertically dashed), 5k-1.4c (horizontally-dashed), and 2k-3.4c (right-dashed). B: 2k-6i (closed columns), 5k-6i (open columns), and 2k-10i (right-dashed).

tration, respectively (14). Mosqueira *et al.* (28) reported that only 20% of the injected dose of 200-nm size LN having a TG core and poly(lactide)-PEG shell was still circulating 4 h after administration. The 70-nm poly(lactide)-PEG micelles of Stolnik *et al.* (29) were also rapidly eliminated, with only 40 and 25% of total dose remaining in the blood 2 and 3 h after iv injection into rats. It must be mentioned that the results of this latter study can be easily compared with our data, since it was performed in the same animal model with similar particle size and injected dose (6 mg/kg).

In conclusion, cholesterol-free LN with a hydrophobic core, controlled sizes and long-circulation times were produced by a simple, solvent-free process. These novel nanocarriers represent drug delivery system alternatives to polymeric nanoparticles, micelles and liposomes. Such LN could prove useful for the solubilization (30) and delivery of lipophilic drugs to solid tumors. Formulation studies with anticancer drugs are currently ongoing, and tumor accumulation of these LN and encapsulated guest molecules is being evaluated in tumor-bearing mice.

ACKNOWLEDGMENTS

This work was partially funded by the Canada Research Chair program. The authors express their gratitude to Nabil Mohamed Khalid for the AFM analysis.

REFERENCES

1. D. C. Drummond, O. Meyer, K. Hong, D. B. Kirpotin, and D. Papahadjopoulos. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *Pharmacol. Rev.* **51**:691–743 (1999).
2. I. Brigger, C. Dubernet, and P. Couvreur. Nanoparticles in cancer therapy and diagnosis. *Adv. Drug Deliv. Rev.* **54**:631–651 (2002).
3. M. C. Jones and J. C. Leroux. Polymeric micelles—A new generation of colloidal drug carriers. *Eur. J. Pharm. Biopharm.* **48**: 101–111 (1999).
4. S. M. Moghimi, A. C. Hunt, and J. C. Murray. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol. Rev.* **53**:283–318 (2001).
5. O. Ishida, K. Maruyama, K. Sasaki, and M. Iwatsuru. Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice. *Int. J. Pharm.* **190**:49–56 (1999).
6. G. Blume and G. Cevc. Molecular mechanism of the lipid vesicle longevity in vivo. *Biochim. Biophys. Acta* **1146**:157–168 (1993).
7. A. Klibanov, K. Maruyama, A. M. Beckerleg, V. Torchilin, and L. Huang. Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposomes binding to target. *Biochim. Biophys. Acta* **1062**:142–148 (1991).
8. R. K. Jain. Delivery of molecular and cellular medicine to solid tumors. *Adv. Drug Deliv. Rev.* **26**:71–90 (1997).
9. A. Sharma, E. Mayhew, L. Bolcsak, C. Cavanaugh, P. Harmon, A. S. Janoff, and J. Bernacki. Activity of paclitaxel liposome formulations against human ovarian tumor xenograft. *Int. J. Cancer* **71**:103–107 (1997).
10. B. B. Lundberg, V. Risovic, M. Ramaswamy, and K. M. Wasan. A lipophilic paclitaxel derivative incorporated in a lipid emulsion for parenteral administration. *J. Control. Rel.* **86**:93–100 (2003).
11. B. Heurtault, P. Saulnier, B. Pech, J. E. Proust, and J. P. Benoit. A novel phase inversion-based process for the preparation of lipid nanocarriers. *Pharm. Res.* **19**:875–880 (2002).
12. B. Heurtault, P. Saulnier, B. Pech, M.-C. Venier-Julienne, J. E. Proust, R. Phan-Tan-Luu, and J. P. Benoit. The influence of lipid nanocapsule composition on their size distribution. *Eur. J. Pharm. Sci.* **18**:55–61 (2003).
13. A. Cahouet, B. Denizot, F. Hindré, C. Passirani, B. Heurtault, M. Moreau, J. J. Le Jeune, and J. P. Benoit. Biodistribution of dual radiolabeled lipidic nanocapsules in the rat using scintigraphy and gamma counting. *Int. J. Pharm.* **242**:367–371 (2002).
14. R. Gref, Y. Minamitake, M. T. Peracchia, V. Trubetskoy, V. Torchilin, and R. Langer. Biodegradable long-circulating polymeric nanospheres. *Science* **263**:1600–1603 (1994).
15. P. S. Uster, T. M. Allen, B. E. Daniel, C. J. Mendez, M. S. Newman, and G. Z. Zhu. Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time. *FEBS Lett.* **386**:243–246 (1996).
16. J. T. P. Derksen, H. W. M. Morselt, and G. L. Scherphof. Processing of different liposome markers after in vitro uptake of immunoglobulin-coated liposomes by rat liver macrophages. *Biochim. Biophys. Acta* **931**:33–40 (1987).
17. P. Moreau, L. Lamarche, A. K. Laflamme, A. Calderone, N. Yamaguchi, and J. de Champlain. Chronic hyperinsulinaemia and hypertension: the role of the sympathetic nervous system. *J. Hypertens.* **13**:333–340 (1995).
18. P. Calvo, B. Gouritin, H. Chacun, D. Desmaele, J. D'Angelo, J. P. Noel, D. Georgin, E. Fattal, J. P. Andreux, and P. Couvreur. Long-circulating PEGylated polycyanoacrylate nanoparticles as new drug carrier for brain delivery. *Pharm. Res.* **18**:1157–1166 (2001).
19. A. Lukyanov, Z. Gao, L. Mazzola, and V. Torchilin. Polyethylene glycol-diacyllipid micelles demonstrate increased accumulation in subcutaneous tumors in mice. *Pharm. Res.* **19**:1424–1429 (2002).
20. K. Sou, T. Endo, S. Takeoka, and E. Tsuchida. Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. *Bioconjug. Chem.* **11**:372–379 (2000).
21. D. B. Fenske, L. R. Palmer, T. Chen, K. F. Wong, and P. R. Cullis. Cationic poly (ethyleneglycol) lipids incorporated into pre-formed vesicles enhance binding and uptake to BHK cells. *Biochim. Biophys. Acta* **1512**:259–272 (2001).
22. J. N. Moreira, T. Ishida, R. Gaspar, and T. M. Allen. Use of the post-insertion technique to insert peptide ligands into pre-formed stealth liposomes with retention of binding activity and cytotoxicity. *Pharm. Res.* **19**:265–269 (2002).
23. V. P. Torchilin, V. G. Omelyanenko, M. I. Papisov, A. A. Bogdanov, V. S. Trubetskoy, J. N. Herron, and C. A. Gentry. Poly-(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim. Biophys. Acta* **1195**:11–20 (1994).
24. G. Blume and G. Cevc. Liposomes for the sustained drug release in vivo. *Biochim. Biophys. Acta* **1029**:91–97 (1990).
25. D. C. Litzinger, A. M. J. Buiting, N. Van Rooijen, and L. Huang. Effect of liposome size on the circulation time and intraorgan distribution of amphipatic poly(ethylene glycol)-containing liposomes. *Biochim. Biophys. Acta* **1190**:99–107 (1994).
26. A. Mori, A. L. Klibanov, V. P. Torchilin, and L. Huang. Influence of the steric barrier activity of amphipathic poly(ethylene glycol) and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo. *FEBS Lett.* **284**: 263–266 (1991).
27. T. M. Allen, C. Hansen, F. Martin, C. Redemann, and A. Yau-Young. Liposomes containing synthetic lipid derivatives of poly-(ethylene glycol) show prolonged circulation half-lives in vivo. *Biochim. Biophys. Acta* **1066**:29–36 (1991).
28. V. C. Mosqueira, P. Legrand, J. L. Morgat, M. Vert, E. Mysiakine, R. Gref, J. P. Devissaguet, and G. Barratt. Biodistribution of long-circulating PEG-grafted nanocapsules in mice: effects of PEG chain length and density. *Pharm. Res.* **18**:1411–1419 (2001).
29. S. Stolnik, C. R. Heald, J. Neal, M. C. Garnett, S. S. Davis, L. Illum, S. C. Purkis, R. J. Barlow, and P. R. Gellert. Polylactide-poly(ethylene glycol) micellar-like particles as potential drug carriers: Production, colloidal properties and biological performance. *J. Drug Target.* **9**:361–378 (2001).
30. A. Lamprecht, Y. Bouligand, and J. P. Benoit. New lipid nanocapsules exhibit sustained release properties for amiodarone. *J. Control. Rel.* **84**:59–68 (2002).