Biodistribution of Long-Circulating PEG-Grafted Nanocapsules in Mice: Effects of PEG Chain Length and Density

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Purpose: To study the pharmacokinetics and biodistribution of novel polyethyleneglycol (PEG) surface-modified poly(rac-lactide) (PLA) nanocapsules (NCs) and to investigate the influence of PEG chain length and content.

Methods: The biodistribution and plasma clearance in mice of different NC formulations were studied with [^{3}H]-PLA. PLA-PEG copolymers were used in NC preparations at different chain lengths (5 kDa and 20 kDa) and PEG contents (10% and 30% w/w of total polymer). *In vitro* and *in vivo* stability were also checked.

Results: Limited [^{3}H]-PLA degradation was observed after incubation in mouse plasma for 1 h, probably because of to the large surface area and thin polymer wall. After injection into mice, NCs prepared with PLA-PEG copolymers showed an altered distribution compared to poloxamer-coated PLA NCs. An increased concentration in plasma was also observed for PLA-PEG NCs, even after 24 h. A dramatic difference in the pharmacokinetic parameters of PLA-PEG 45–20 30% NCs compared to poloxamer-coated NCs indicates that covalent attachment, longer PEG chain lengths, and higher densities are necessary to produce an increased half-life of NCs *in vivo*.

Conclusions: Covalently attached PEG on the surface of NCs substantially can reduce their clearance from the blood compartment and alter their biodistribution.

KEY WORDS: nanocapsules; poly(rac-lactide)-poly(ethylene glycol) copolymers; plasma stability; biodistribution; plasma clearance; PEG chain length.

INTRODUCTION

The rapid uptake of colloidal drug carrier systems by the mononuclear phagocyte system (MPS) after intravenous administration is the major barrier preventing their use for delivering drugs to sites other than Küpffer cells in liver and macrophages in the spleen (1). A large amount of work has been performed over the previous 10 years to optimize carriers that have a longer half-life in the bloodstream and altered biodistribution, allowing them to reach other cells and tissues, such as circulating cells themselves, solid tumors, and inflammatory sites (1,2). In particular, poly(ethylene glycol) (PEG) has been used successfully to modify the surface of liposomes, thereby dramatically increasing their circulating half-life (2).

To apply this strategy to safe biodegradable polymeric carriers, poly(lactide-glycolide) (PLGA) nanospheres have been surface modified by coating with amphipathic triblock and star-shaped copolymers, such as poloxamers and poloxamines (3–5). However, as well as the problem of desorption in the circulation, the safety aspects of the intravenous administration of these surfactants remain an important issue to consider because their biologic effects are not negligible and not well understood (6). The synthesis of these amphiphilic PEG-bearing polymers allows the direct preparation of PEG nanospheres, ensuring the stability of the PEG coating layer, because the PEG blocks are covalently linked to the nanosphere core. Furthermore, PEG is non-toxic, and chains shorter than 30 kDa readily are eliminated from the body by renal filtration (7); thus, this molecule can be considered completely biocompatible. Gref *et al.* reported that nanospheres prepared from diblock PLGA-PEG copolymers have considerable extended blood circulation times and reduced liver accumulation in mice, and that these effects were accentuated with increasing molecular weights of the PEG component from 5,000 to 20,000 Da (8). As in the case of liposomes, the altered biodistribution may be attributed to reduced opsonisation of these carriers resulting from the steric stabilization afforded by a hydrophilic and flexible PEG layer at their surface (8–10).

Oil-based colloidal carriers, such as nanoemulsions, have also been surface-modified (11,12). However, very little work has been done concerning the surface modification of vesicular polymeric colloidal drug carriers, such as nanocapsules (NCs). The main advantages of NCs include the possibility of transporting a highly concentrated active molecule within the oil core and their stability due to their polymeric wall with greater protection against enzymatic degradation compared with nanoemulsions, leading to reduced toxicity as well as changes in the pharmacokinetic profile of drugs (13,14). The few examples found in the literature obtained surface modification by adsorption of PEG-containing surfactants (poloxamers), which led to increased concentration of an associated drug in murine tumors (15). Recently, we have described the preparation of a novel surface-modified NCs bearing PEG covalently grafted to a biodegradable PLA polymer (16,17). Detailed investigations were performed to determine the effect of varying the length of PEG chains and their density at the surface on the interactions of NCs with macrophages. The covalent attachment of these PEG chains to the hydrophobic poly(rac-lactide) polymer allowed the preparation of NCs, which showed a dramatic reduction in their interactions with macrophages *in vitro* for at least 18 h, depending on the PEG chain length and density. As a control, we prepared NCs from a PLA homopolymer. However, because these NCs had a tendency to aggregate on storage, they were stabilized by adding poloxamer 188 to the aqueous phase. This surfactant could also modify the surface properties by adsorption. In fact, we observed that in cell culture, although the uptake was reduced compared to "naked" PLA NC at

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high concentration, this property was lost at higher dilution, suggesting reversible adsorption (16).

Thus, in the present work, the main goal was to study the *in vivo* distribution of these surface-modified biodegradable NCs prepared from PLA-PEG copolymers with a range of PEG chain length and content. PLA NCs stabilized with poloxamer 188 were used as controls. To validate the results, the stability of the nanocapsules in plasma was also studied.

MATERIALS AND METHODS

Soy phospholipids (lecithin with ~70% PC) Epikuron 170®, were purchased from Lucas Meyer (France) and Poloxamer 188 (Synperonic F68) from ICI (France). Miglyol 810N (caprylic/capric triglyceride) was kindly provided by Hülls (France). Poly (rac-lactic acid), Mw 42000 (PLA_{50}) was supplied by Phusis (France). PLA_{50}-PEG 45-5 (PLA_{50} molecular mass (Mm) 45,000 and PEG Mw 5,000), PLA_{50}-PEG 45–20 (PLA_{50} Mw 45,000 and PEG Mw 20,000) were synthesized and characterized as described previously (18). The solvents used were analytical grade, and all other chemicals were commercially available reagent grade. Water was purified by reverse osmosis (MilliQ, Millipore® St-Quenten-en-Yuelines, France).

Preparation and Characterization of Radiolabeled NCs

NCs were prepared by the method described by Fessi *et al.,* based on interfacial polymer deposition following solvent displacement (19). Poly(rac-lactic acid) (PLA_{50}) Mw 42,000 was used with poloxamer 188, a hydrophilic surfactant to prepare nonsurface-modified NCs, referred to as PLA-POLOX NC. Surface-modified NCs were prepared using the diblock polymers (PLA_{50}-PEG 45-5 or PLA_{50}-PEG 45-20) and PLA_{50} so as to obtain PEG contents of 10 or 30% of PEG w/w with respect to total polymer in the acetone phase, with lecithin and Miglyol 810N as above. No poloxamer was added to the aqueous phase in this case. The surface-modified NCs are referred to as PLA-PEG x y% NC, where x is the PEG chain length in kDa and y the percentage of PEG by weight in the copolymer.

Briefly, 6 mg of polymer were dissolved in 2 ml of acetone containing 7.5 mg of soy lecithin (Epikuron® 170), 25 μl of Miglyol 810N. This organic solution was poured into 4 ml of external aqueous phase under agitation containing 0.375% w/v of Synperonic® F68 only in the case of PLA-POLOX NC, and the solvent was evaporated to 1 ml under reduced pressure. The size of the nanocapsules was determined by quasi-elastic light scattering (QELS), with a Nanosizer (Coulter model N4 Plus, Coulter Electronics Inc., Hialeah, FL, USA). Radiolabeled NCs were prepared including a trace amount of ^{3}H-PLA(rac-lactide) Mw 57,000 polydispersity index (Mw/Mn = 2–2.1), specific activity 4.181×10^{7}Bq/mg (^{3}H-PLA), synthesized and purified as described (20) from rac-lactide previously tritiated in tertiary proton (CH groups) by high-temperature solid-state catalytic isotopic exchange. In some experiments a second marker, n-hexadecane-1-^{14}C (Mw 226.4, specific activity 1.517×10^{8}Bq/mmol; Sigma, France) was added to the organic phase. The percentage of radiolabel incorporation in NC preparations was determined by size-exclusion chromatography as described below.

In Vitro Stability of NCs in Murine Plasma

The stability of NCs, which were prepared with trace amounts of [^{3}H]-PLA in the polymeric wall and [^{14}C]-hexadecane in the internal oil phase, was determined in the presence of 50% murine plasma at 37°C. One-hundred microliters of NCs were incubated with 100 μl of freshly collected murine plasma at 37°C for 1 h. To isolate NCs from murine plasma components, the mixture was loaded on a column (Biogel A1.5, 2.0 × 22.5cm, 100–200 mesh; fractionation range 10,000–1,500,000Da) and eluted with isotonic phosphate-buffered saline (PBS, pH 7.4) at 1.1 ml/mim using a peristaltic pump (Microperpex®, LKB Bromma, France). The eluate was analyzed by a Shopex RI differential refractometer (Showa Denko, Japan) coupled to an integrator (Shimadzu, Japan). Aliquots of each fraction (100 μl) were assayed for ^{3}H and ^{14}C by scintillation counting in 3 ml of UltimaGold® scintillation cocktail. For dual-label studies, channel windows were set to collect ^{3}H and ^{14}C cpm simultaneously. The elution profile was then constructed. The degradation was estimated by comparing the percentage of radiolabel co-eluting with the NCs before and after incubation in murine plasma. The particle size was determined by light scattering after NC preparation and again after incubation in plasma and passage through the Biogel column. The elution profiles of the plasma itself, freshly prepared NCs, and NC incubated in the presence of plasma were compared. Degradation kinetics were studied by incubating NCs labeled only with [^{3}H]-PLA in 70% murine plasma for different times followed by ultrafiltration/centrifugation technique in Ultrafree® Units (cut-off 0.1 μm; Millipore, France).

Preparation of Degraded PLA

Two milliliters of NaOH 2N were added to 120 μCi (4.44MBq) of [^{3}H]-PLA (106 μg) in acetone (120 μl). The mixture was incubated under agitation at 70°C for 20 h. The solution was adjusted to pH 7.2 with HCl 1N, filtered in a 0.2 μm sterile filter, and the volume adjusted with PBS pH 7.4 to obtain 20μCi/ml. The soluble degradation products of poly-(lactic acid) were then injected into mice (2μCi/100 μl) to follow their behavior and clearance profile *in vivo* in the same way as for NCs.

Biodistribution Studies in Mice

To assure that all radioactivity administered to mice was associated with NCs and thus truly reflected carrier distribution, [^{3}H]-PLA-radiolabeled NCs were purified by size-exclusion chromatography just before injection. The NC fractions detected by differential refractometry were pooled, filtered on a 0.45-μm sterile filter, and adjusted to the concentration required. Six to seven week-old Swiss CD1 female mice weighing 21–25 g (Charles River, France) were injected intravenously via the retro-orbital sinus with 150 μl of radiolabeled NC suspension diluted in isotonic PBS (5 mg/kg of polymer) and 1 μCi (37KBq) of ^{3} per mouse. At the indicated time intervals, 5, 30, 90, 240, 360, min and 24 h post-injection, the mice were anesthetized by intraperitoneal injection of 100 μl of sodium pentobarbital (40 mg/Kg) and bled by cardiac puncture. Blood (approximately 1 ml) was collected in heparinized microcentrifuge tubes and liver, spleen, lungs, heart, kidneys, one femur (left hind leg), fat

tissue, and muscle were removed. The blood, plasma, and all tissues were weighed, dissolved with Soluene 350® (1 ml), bleached with 0.2 ml of H_2O_2 (0.4 ml in blood samples) and scintillation medium (IonicFluor®, Packard) was added before counting the radioactivity in a liquid scintillation counter (Beckman, France). To determine the amount of total radioactivity in whole blood and plasma, a total blood volume and plasma volume per mouse of 7.5% and 4.9% of body weight, respectively, were assumed (21). The results were expressed as a percentage of the injected dose and are the mean ± standard deviation of four mice per point. NCs labeled with ^{3}-PLA served as a marker to determine the distribution of NCs in the various tissues. Total percentage of injected dose per organ and radioactivity per mg of tissue were calculated.

Pharmacokinetics and Statistical Analysis

To compare the different formulations, the mean area under the curve (AUC) of [^{3}]-PLA NC concentrations in plasma and organs was calculated by the trapezoidal method during the experimental period ($AUC_{[0-24]}$). The extrapolated area ($AUC_{[t-\omega]}$) was calculated by dividing the last experimental concentration (C_t) by the rate constant of elimination (K) because this area represented approximately 1% of the experimental AUC, it was neglected in our calculations. Thus, the overestimated plasma clearance (Cl) was calculated from the equation (1) and the mean residence time (MRT) from the equation (2):

$$Cl = Dose_{iv}/AUC_{0-24} \quad (1)$$

$$MRT = I_{0-6}\, t\, C_n \cdot dt_n)/AUC_{0-6} \quad (2)$$

The MRT was calculated up to the experimental point at 6 h; that is, for the period during which none of the NC preparations reached the level of degraded lactic acid (see Fig. 3B). All data above were expressed as mean value and the concentrations in each organ as a mean value × SD (n = 4 mice per time point).

RESULTS

Characterization of NCs

The NC formulations used in this study have already been characterized in the previous study (17). The main results are summarized in Table I. The mean diameter of the NCs was around 200 nm, and the polydispersity was always less than 0.3 (Table II); therefore, the preparations could be considered as monodisperse. The negative zeta potential was only slightly masked by the presence of the longer PEG chains at high density because the soy lecithin present in the formulation contributes to the surface properties. The complexity of the NC system means that several forms could be present at the same time; NC, nanospheres formed from polymer without an oily core and liposomes formed from excess lecithin. Nanospheres could be separated from NCs and liposomes by density gradient centrifugation. A band of higher density (around 1.1) corresponding to nanospheres was found in the preparations containing more than one polymer : PLA-POLOX NC (containing PLA and poloxamer) and PLA-PEG 20 10% (containing a mixture of PLA-PEG co-polymer and PLA), whereas those prepared from PLA-PEG copolymers alone yielded a single band at 1.018–1.035 consistent with NC (17). As far as interactions with biologic systems were concerned, the NCs with 30% of PEG of 20-kDa chain length gave the lowest activation of complement and the smallest association with the J774A1 macrophage-like cell line (16). Despite the fact that the theoretical PEG chain density at the surface was similar for PLA-PEG 5 10% NC and PLA-PEG 20 30% NC (see Table III), the shorter chain length yielded less marked "Stealth" properties. It was, therefore, interesting to study the pharmacokinetics and distribution of these NC *in vivo*. To follow the particles easily, radioactively labeled NCs were prepared.

Efficiency of Labeling and *In Vitro* Stability of NCs in Mouse Plasma

The results of the size-exclusion chromatography (SEC) of different NC formulations are shown in Fig. 1 and Table II. They indicate that not all the [^{3}H]-PLA added to the preparation was associated with the NCs because a part of the radioactivity is eluted in the total volume (fractions 35–50), corresponding to the aqueous phase of NC preparation as detected by the negative peak in differential refractometry and therefore having a hydrodynamic radius equivalent to a protein of 10 kDa or less. This probably represents low molecular weight polymers or the products of radiolysis during polymer storage. Therefore, the [^{3}H]-labeled NCs were purified by this technique before administration to mice. The yield of ^{3}H labeling was about 55% for all NC formulations, whatever the composition, with or without PLA-PEG copolymers. In contrast the oil phase label, 1-[^{14}]n-hexadecane was

Table I. Characteristics of the NC[a] Preparations

NC Designation	Polymer(s) used (molar ratio)	Poloxamer 188	PEG % w/w	ζ Potential (mV)[b,c]	Density gradient centrifugation[c]	C3 cleavage[c]	Relative uptake by J774A1 cells[d]
PLA-POLOX	PLA_{50}	+	–	–51 ± 0.2	Major band at 1.035, minor band at 1.102	+++	100
PLA-PEG 5 10%	PLA_{50}-PEG 45-5	–	10%	–51 ± 1.1	Single band at 1.035	+++	39
PLA-PEG 20 10%	PLA_{50}-PEG 45-20:PLA_{50} (1:2)	–	10%	–46 ± 0.6	Major band at 1.035, minor band at 1.102	ND	27
PLA-PEG 20 30%	PLA_{50}-PEG 45-20	–	30%	–38 ± 1.1	Single band at 1.018	+	8

[a] NC = nanocapsule, PEG = polyethyleneglycol, PLA = poly (rac-lactide), ND = not determined.
[b] Mean ± standard deviation (n = 4) reported by the instrument on a typical preparation.
[c] Data taken from Ref. 17.
[d] Data taken from Ref. 16.

Table II. Composition, Size, and Stability of the NCs[a] Radiolabeled with [^{3}H]-PLA Polymer and [^{14}C]-1-n-hexadecane, before and after Incubation in Murine Plasma at 37°C

NC formulation	Size (nm)[b] and polydispersity index after SEC column	Radioactivity recovery after size exclusion chromatography in different pools(%)[c]						Total release (%)		Additional release in plasma(%)	
		^{3}H F14-20	^{14}C F14-20	^{3}H F21-34	^{14}C F21-34	^{3}H F35-50	^{14}C F35-50	^{3}H	^{14}C	^{3}H	^{14}C
PLA-POLOX	136 (0.18)	54	97	3	3	44	1	46	3		
PLA-POLOX + plasma[d]	131 (0.07)	37	86	4	12	59	2	62	14	16	10
PLA-PEG 5 10%	170 (0.25)	55	96	5	5	39	1	45	7		
PLA-PEG 5 10% + plasma[d]	167 (0.20)	46	90	4	12	51	2	54	13	10	6
PLA-PEG 20 10%	176 (0.28)	55	95	2	3	43	2	45	5		
PLA-PEG 20 10% + plasma[d]	174 (0.18)	46	84	4	14	51	1	54	16	9	11
PLA-PEG 20 30%	197 (0.21)	57	93	5	6	38	1	43	8		
PLA-PEG 20 30% + plasma[d]	196 (0.12)	40	86	4	11	56	3	60	14	17	6

[a] NC = nanocapsule, PLA, poly (rac-lactide), SEC = size-exclusion chromatography.
[b] Monodisperse samples (P.I. below 0.3).
[c] The total radioactivity recovery from the column was 86–99% in all experiments.
[d] NC injection (100 μl) on the top of the column after incubation at 37°C for 60 min 1:1 NC in murine plasma.

associated with NCs to a higher extent (93–96%), as estimated by the total radioactivity in fractions 14–21. Once the NCs were prepared, the percentage of ^{3}H and ^{14}C co-eluted with NC was not changed after 48 h of storage at 4°C (data not shown).

The stability of different NC formulation was tested after a 60-min incubation in 50% mouse plasma at 37°C without previous purification to prevent excessive dilution on the SEC columns. We first examined the effect of concentration and of PEG molecular weight of diblock PLA-PEG polymers on NC stability in murine serum. No major differences between different preparations could be detected. The size of NCs were not significantly changed, and the polydispersity index decreased slightly after incubation in plasma and SEC chromatography, showing the absence of disintegration or aggregation. After this incubation, an additional release of [^{3}H]-PLA label in low molecular weight form (eluted in the total volume) was observed, which could be interpreted as release of less tightly associated oligomers or even partial PLA wall degradation. The increase in non-associated [^{3}H]-PLA was about 16% for PLA-POLOX and PLA-PEG 20 30% NC and slightly lower for PLA-PEG 5 10% NC and PLA-PEG 20 10% NC (Table II). However, most of 1-[^{14}C]-n-hexadecane remained associated with the particles that eluted in the void volume of the column, i.e., with a diameter larger than 150 nm (Fractions 14–21 in Table II). In contrast to ^{3}H-PLA, the released ^{14}C was recovered in the fractions 21–34, which could be identified as plasma proteins by comparison with the refractometry profile obtained from plasma alone. Figure 2 shows the kinetic of degradation analyzed by a centrifugation/ultrafiltration technique and confirms that the [^{3}H]-PLA associated with NCs were only slightly degraded during a 24-h incubation in 70% murine plasma, without any significant difference between the formulations ($P > 0.05$ for each time point). Therefore, we considered that if ^{3}H-labeled low molecular weight material was removed from the preparation by SEC before injection, these NCs had sufficient stability to allow us to follow their fate *in vivo* by counting the tritium label associated with the NC wall. We decided not to follow [^{14}C]-labeled n-hexadecane in the *in vivo* study because we

Table III. Pharmacokinetic Parameters (Plasma) of [^{3}H]-PLA-Labeled Solution and NC[a] after Intravenous Injection in Mice: Effect of PEG Distance (***D***) and Chain Length

	[^{3}H]-PLA hydrolyzed	[^{3}H] PLA-NCs			
		PLA-POLOX	PLA-PEG 5 10%	PLA-PEG 20 10%	PLA-PEG 20 30%
Size ± Standard deviation (nm)[b]	–	138 ± 45	155 ± 58	176 ± 49	197 ± 66
D [PEG-PEG] (nm)[c]	–	ND[d]	4.46	8.40	4.51
$AUC_{0-24\ plasma}$ (%Dose.min)	5730	1164	10325	4532	17497
Cl (min^{-1})	0.018	0.086	0.010	0.022	0.006
$MRT_{0-6\ plasma}$ (min)	ND	70.4	139.9	114.8	140.7
$AUC_{0-24\ blood}$ (%Dose.min)	5725	2175	10710	5917	11397
$AUC_{0-24\ liver}$ (%Dose.min)	3125	12553	5802	10341	5024
$AUC_{0-24\ spleen}$ (%Dose.min)	217	759	482	434	436
$AUC_{0-24\ kidneys}$ (%Dose.min)	781	368	751	625	891

[a] NC = nanocapsule, PEG = polyethyleneglycol, PLA = poly (rac-lactide).
[b] Mean ± standard deviation reported by the instrument ($n = 4$) for a single preparation.
[c] Calculated as described in reference 16.
[d] Not determined because poloxamer 188 is reversibly adsorbed; AUC_{0-24} experimental area under the curve, dose = 150 μl (1 μCi); Cl = clearance, MRT = mean residence time.

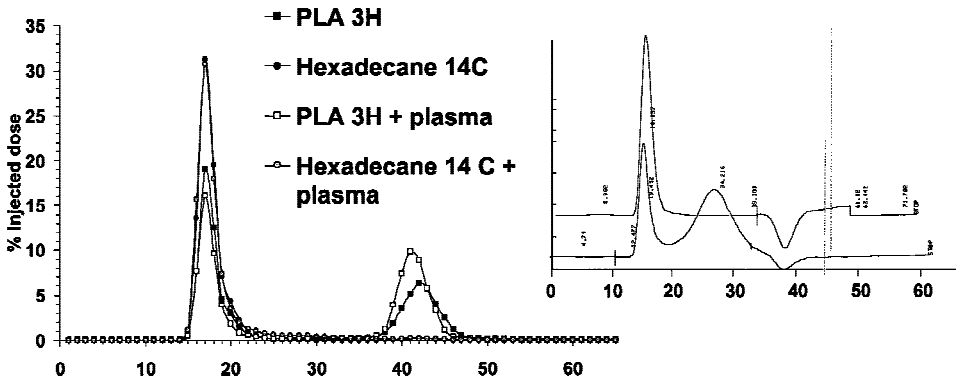

Fig. 1. Label incorporation and stability of PLA-POLOX NC nanocapsules in 50% murine plasma. Elution profile on Biogel column of radioactive fractions [^{3}H]-PLA, squares, and [^{14C}]-n-hexadecane, circles (% of injected dose in column versus elution volume) before (closed symbols) and after (open symbols) incubation in murine plasma (1:1) at 37°C. The insert shows the refractometry profiles: upper trace before and lower trace after incubation.

wanted to concentrate on the carrier itself rather than the encapsulated drug.

Clearance of Hydrolyzed PLA and PLA NC Degradation *In Vivo*

The PLA in the NC wall is expected to be degraded *in vivo* and the rate of elimination of ^{3}H radioactivity will be a function of the rate of degradation and the rate of elimination of the degradation products. To estimate the latter, a sample of fully hydrolyzed [^{3}H]-PLA was administered intravenously. In this case, the radioactivity in the plasma decreased very rapidly and was evenly distributed among the major organs of the body, as shown in Fig. 3A. Five minutes after injection, only 6% of the total injected radioactivity was found in plasma and 3% in liver. The plasma clearance of the residual amounts of degraded ^{3}H-PLA (or lactic acid) from the mice was very slow. The data expressed as Bq/mg for each organ indicate that at 5 min post-injection the radioactivity was approximately the same in all vital organs with slightly increased concentrations in plasma and kidneys, indicating that free lactic acid is probably eliminated by urinary excretion (Fig. 3A). Soluble degradation products were not accumulated in liver and spleen, and their concentration in these organs seemed to be related to the blood flow and was almost constant from 5 min to 24 h. Furthermore, up to 24 h post-injection, the radioactivity in all organs decreased very slowly with higher concentrations in plasma and in muscle.

Figure 3B shows that after NC administration, the elimination of radioactivity from certain key organs of the body was slower than that for hydrolyzed PLA and occurred at almost same rate for all formulations. However, the recovery of radioactivity from PLA-POLOX NC declined sharply between 3 and 6 h, at which time it was similar to that of hydrolyzed PLA and lower than that of NC with covalently

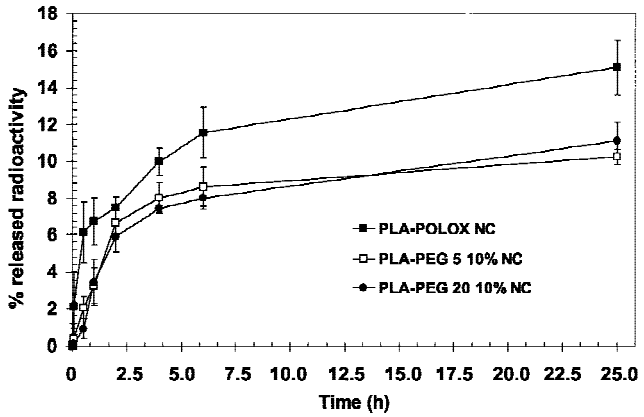

Fig. 2. Kinetics of [^{3}H]-PLA release from PLA-NCs containing PEG after incubation in 70% murine plasma at 37°C and separation on an 0.1-μm ultrafiltration membrane (Millipore®) by centrifugation. The results are calculated as the difference between the percentage of radioactivity in the ultrafiltrate before and after incubation.

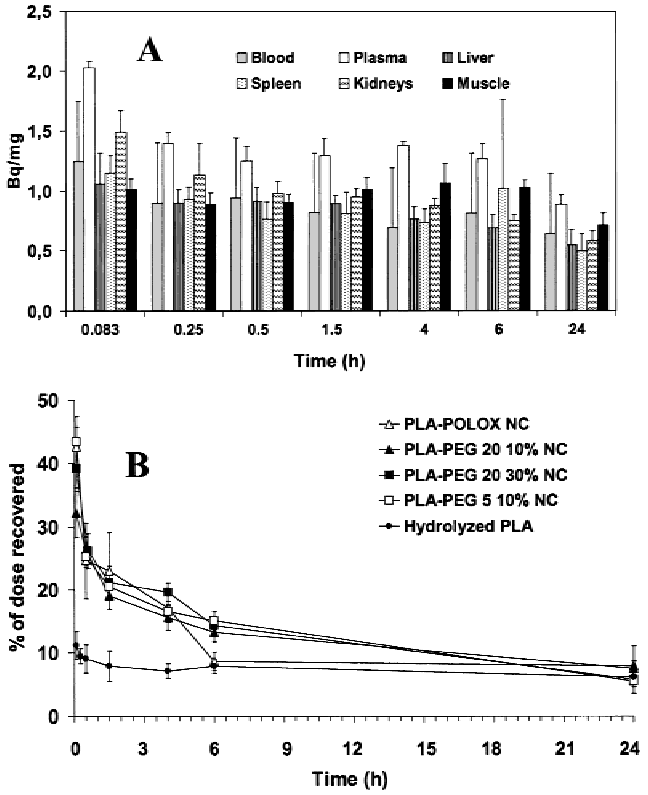

Fig. 3. Organ distribution of hydrolyzed [^{3}H]-PLA polymer in mice in **(A)** and differences in total radioactivity clearances of [^{3}H]-PLA NC with different formulations from certain key organs **(B)**. The total radioactivity in the body was calculated as sum of radioactivity in blood, liver, spleen, kidneys, heart, and lungs.

linked PEG. At 24 h, the recovery of radioactivity in the major organs was similar for all groups. Although no measurements were made between 6 h and 24 h, these data suggest that PEG covalently grafted at the NC surface reduces the rate of degradation of NCs *in vivo.*

Plasma Pharmacokinetics and Biodistribution of NCs

The plasma pharmacokinetic parameters obtained from the various NC formulations and from hydrolyzed PLA are summarized in Table III. Plasma and liver profiles are shown in Fig. 4A and B, respectively. The mean ^3H concentrations in various organs after intravenous administration to mice are illustrated in Fig. 5. PLA-POLOX NC were readily cleared from the plasma and concomitantly accumulated in the MPS organs (liver and spleen, 31% and 1%, respectively) 5 min after injection. In the case of PLA-PEG 20 30% NC, the concentrations of radioactivity in the plasma were significantly higher than those for PLA-POLOX NC and for the hydrolyzed PLA solution and the clearance was reduced (Table III). Furthermore, the amount of radioactivity in the liver was dramatically reduced throughout the period of the experiment (24 h). After 4 h, only 4% of the injected dose was associated with the liver compared to 14% for the PLA-POLOX NC and even at 24 h the level of radioactivity in the liver was lower for all PLA-PEG NC. The differences in distribution of the NC formulations are emphasized in Fig. 6, which shows the ratio of the ^3H concentration in the liver to that in the plasma at various time intervals. PLA-POLOX NCs show rapid accumulation of radioactivity in the liver

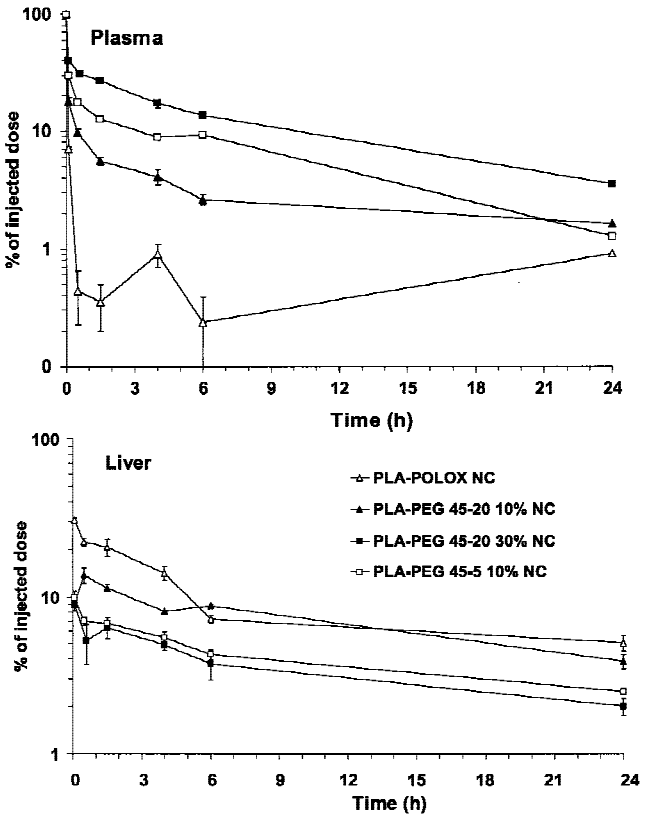

Fig. 4. Plasma and liver [^3H] concentration–time profiles after intravenous injection of 150 μl (37 kBq) of NC suspension (5 mg/kg) in mice (mean ± SD, $n = 4$ per time). Symbols are defined in the liver panel.

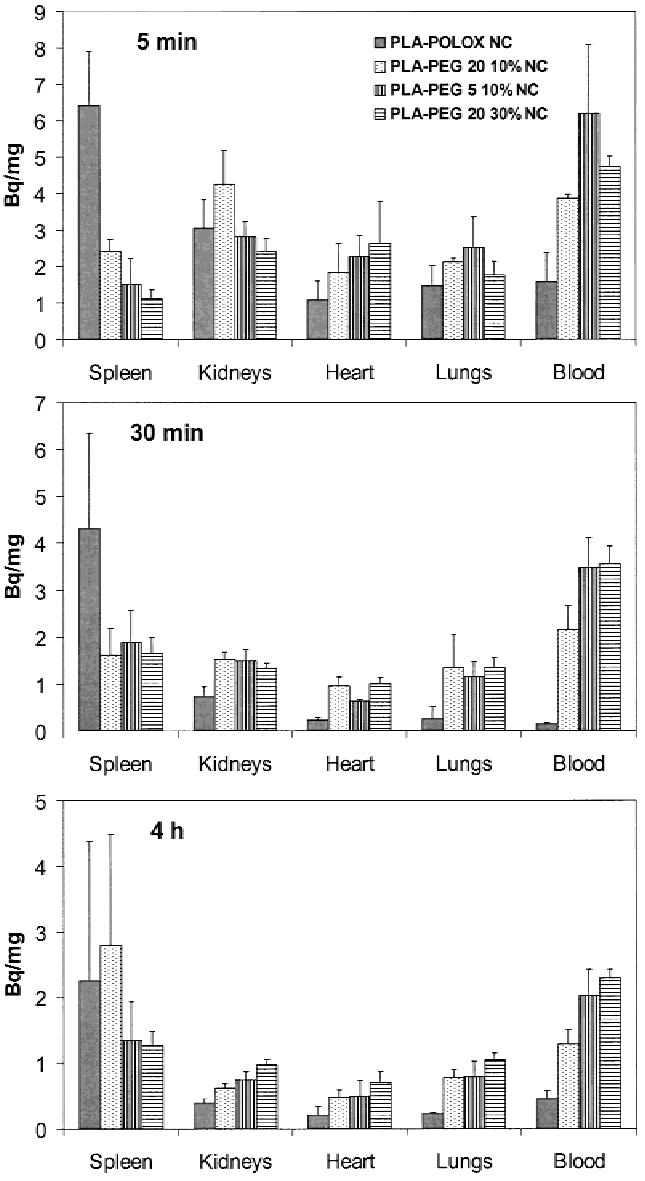

Fig. 5. The effect of poloxamer coating and covalently linked PEG chain length and content on radioactivity deposition in different tissues (Bq/mg) determined after intravenous injection of nanocapsules in mice (dose 37 kBq [^3H]).

during the first 90 min, followed by a gradual decline. The profile for PLA-PEG 45–20 10% NC has the same time-course as PLA-POLOX NC, but liver uptake is reduced. On the other hand, for PLA-PEG 45-5 10% NC, uptake seems to be delayed until 24 h, and for PLA-PEG 45–20 30% NCs, the liver/plasma ratio rises only slowly to equal the value for hydrolyzed PLA at 24 h. Thus, a clear advantage of grafted over adsorbed PEG for avoiding liver uptake was observed. For covalently linked PEG, differences in chain length and PEG distance at the NC surface influenced the clearance of the different formulations. At the same PEG distance (***D***), the higher chain length (20,000) resulted in a reduced clearance compared to 5,000 chains. However, at the same chain length (20,000) a smaller distance led to lower clearance. Figure 5 shows that the radioactivity associated with organs, such as heart and lungs, for PLA-PEG NC is higher than that for

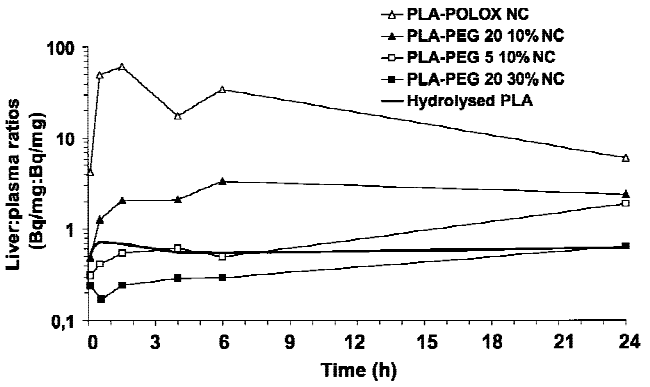

Fig. 6. Ratio between mean liver and plasma [^{3}H] concentrations (Bq/mg) for different preparations as a function of time.

PLA-POLOX NC. This indicates that PEG-grafted NCs circulate longer and reach organs other than the MPS.

DISCUSSION

The biodistribution and plasma clearance of biodegradable, surface-modified NCs prepared from PLA-PEG copolymers with a range of PEG chain length and content. NCs differ from the nanospheres previously prepared from the same co-polymers by their oil-filled core, which can incorporate a large payload of a hydrophobic drug. The presence of the polymeric membrane gives NCs an advantage over micelles and emulsions, which tend to disintegrate or aggregate respectively upon dilution in plasma. Indeed, NCs remained essentially unchanged in terms of size after incubation in murine plasma (Table II).

In this study, the pharmacokinetics and biodistribution were assessed using a radioactive tracer in the polymeric wall. The results were validated by the stability of NCs in plasma and by following the fate of PLA degradation products *in vivo*. "Stealth®" systems should achieve a compromise between ultimate biodegradability and sufficient stability in the blood compartment to allow controlled drug release. The *in vitro* stability studies undertaken in this work showed that some degradation of PLA in NCs occurs during 60 min of incubation. This degradation rate is faster than that of nanospheres prepared from a similar polymer (9), which can be explained by the fact that in NCs all the polymer is present in a thin layer at the surface, providing a large area of interface with the blood. Nevertheless, the fact that ^{3}H-PLA release in presence of plasma was limited even over a 24-h period means that this marker can be considered a reliable reflection of NC distribution.

To address the question of PLA degradation *in vivo* and the influence of degradation products on the pharmacokinetic profiles of PLA-NC, we investigated the profile of elimination of previously hydrolyzed PLA after intravenous administration. The main degradation mechanism of PLA and of PLA-PEG diblock polymers is random hydrolytic scission, amorphous rac-PLA polymer being degraded faster than semicrystalline L- or D- PLA (22). PLA degradation *in vivo* would depend on the amount of polymer administered, the ability of the different tissues to clear degradation products, and the rate of degradation, which in turns depends on the initial molecular weight, copolymer composition, and the morphology of the particles (22,23). The final degradation product of PLA, lactic acid, is a physiologic molecule that is actively metabolized by cells to pyruvate, which can enter the Krebs cycle as acetyl CoA and can then be catabolized to carbon dioxide and water or be used for the synthesis of other biomolecules (24,25). Indeed, in our studies, a small part (~5%; about 85 nanomoles of lactic acid) of the [^{3}H]-radioactivity remained in the body, probably because it had entered metabolic pathways and accumulated in tissues. The ^{3}H-lactic acid generated from the polymer used here is probably eliminated via the kidneys in its intact form or as tritiated water. On the other hand, when radioactivity from blood and five major organs was pooled, all PLA NC showed a similar rate of elimination *in vivo,* different from that of hydrolyzed PLA as shown in Figure 3B. These results indicate that the difference observed in the plasma residence time for different NC formulations reflects their susceptibility to removal from the circulation by phagocytic cells rather than a difference in their degradation rate. The high plasma clearance of PLA-POLOX NC compared with hydrolyzed PLA is counterbalanced by high liver uptake and confirms the hypothesis that this organ is the major site responsible for NC clearance and degradation, probably by the action of Küpffer cells and other macrophages. It should be noted that the quantity of NCs administered was kept low (5 mg/kg total PLA) and was identical for all formulations, to avoid artifacts due to saturation of the MPS (26).

Covalent attachment of PEG to the NC surface led to significant changes in the body distribution of the particles, especially with PLA-PEG 20 30% NC. The plasma clearance profiles for PLA-POLOX NC, PLA-PEG 20 10% NC, 30%, and PLA-PEG 5 10% NC are shown in Fig. 4A. The experimental AUC (plasma) and the mean residence time for all PLA-PEG NC are higher than for the PLA-POLOX, indicating that the covalent attachment of PEG to the NC surface is important for prolonging the residence time *in vivo* and maintaining the reduced association with MPS organs. This effect was pronounced even at 24 h after injection. Higher concentrations of PLA-PEG NC were observed in plasma and outside the MPS system (heart, kidneys, muscle, etc). PLA-PEG NCs were associated with the spleen to a lesser extent than PLA-POLOX (Fig. 5). At 5 minutes after injection, 40% of injected dose of PLA-PEG 20 30% NC was in the plasma and only 8% in the liver compared with 7% and 30%, respectively, for PLA-POLOX NC. Thus, although, PLA-POLOX NC probably have poloxamer adsorbed on the surface after preparation, this does not confer significant long-circulating properties, probably because the surfactant is displaced on dilution in the blood and by competition with opsonins. This is consistent with our observations *in vitro* (16).

The rate of NC removal by the liver seems to determine the clearance (Table III). It is interesting to correlate the clearance values with PEG density at the NC surface, because with increasing ***D*** (distance between two PEG chains) the clearance increases for the PLA-PEG 20 NC chain length; however, at the same PEG distance, the PEG chains of 20 kDa length are more effective than the PEG chains of 5 kDa length in prolonging the plasma residence time. PLA-PEG 20 30% NCs show the longest circulation time and the lower liver uptake (Fig. 4). These results are in accordance with our previous results concerning complement interaction and mac-

rophage uptake *in vitro* (16), suggesting that similar mechanisms are involved.

It is interesting to compare the *in vivo* behavior of these NCs with nanospheres obtained previously from similar PLGA-PEG copolymers (8). In both cases, the blood circulation time increases as the chain length increases for the same PEG density. It is important to consider that the diameter of PLA-PEG NCs (150–200 nm) is higher than that of PLGA-PEG nanospheres (80–150 nm) and that NCs have a lower PEG content: only 1.7–5.3% of PEG w/w of dry NC suspension compared to 10% w/w in PLA-PEG nanospheres. Furthermore, the distances, ***D***, between end-attached PEG chains, which can be achieved in the NC system, are superior to the threshold value of about 2 nm found to be necessary to provide steric repulsion and long-circulating properties in PLA-PEG nanospheres (9,27). In spite of this, PLA-PEG NCs show long-circulating properties, especially when 20-kDa PEG chains are present. In fact, the liver association of PLA-PEG 20kDa 30% NC was 2- to 3-fold lower in all time points than that obtained with PLGA-PEG 20 kDa nanospheres (8). These "Stealth" properties observed at relatively low PEG densities could be attributed to the presence of lecithin in the NC formulation. Zeta potential measurements indicate that this lecithin plays a role at the NC surface. In NCs without grafted PEG, increasing the proportion of Epikuron 170 in the formulation leads to a more negative zeta potential. On the other hand, the use of a higher-grade lecithin with fewer charged impurities yields a less negative zeta potential (28). Similarly, in NCs bearing covalently linked PEG, when the lecithin content was reduced from the 0.75% (w/v) used in the preparations studied here to 0.30% (w/v) (the minimal necessary to obtain NC) the absolute values of zeta potential were reduced; for example that of the PLA-PEG 20 30% formulation was about –5 mV in the presence of 0.30% (w/v) lecithin (17). Furthermore, comparisons of complement activation by different formulations suggest that the presence of lecithin reduces C3 cleavage (17).

A question arises about the biologic fate of 20-kDa PEG chains. It has been demonstrated that the urinary clearance of PEG changes abruptly at a molecular weight of around 30 kDa, suggesting that this value represents the glomerular filtration cut-off for this nonionic polymer (7). Thus, the use of PEG molecular weights of 20 kDa in the NC composition is justified, considering their better efficiency in reducing clearance of NCs *in vivo*. Nevertheless, the PEG density at the NC surface could be further optimized by using other amphipathic biodegradable polymers with more than one PEG chain per PLA chain to obtain PEG distances as small as those used in preparation of Stealth® liposomes (2) and nanospheres (9,27). Future experiments with different PEG contents are necessary to determine the PEG threshold value for affecting NC circulation time. Other biodegradable polymeric blocks could be used order to reduce the rate of degradation *in vivo,* for example as poly(L-lactide) or poly-ε–caprolactone. Furthermore, it would be advantageous to prepare NC smaller than 200 nm, this being considered optimal for Stealth® systems to prevent a filtering mechanism in the spleen and an increased uptake by macrophages (9,26), although this would reduce the payload per particle.

PEG surface-modified liposomes (Stealth®) effectively alter the biodistribution and stability of drugs in the body, although they are rarely able to incorporate more than 5 mol%, typically 1–2%, compared to the total lipid, of a hydrophobic drug into the bilayer (2). Thus, NCs could be useful alternative for this type of drug, considering their ability to encapsulate amounts as high as one-third of the total NC weight. This drug payload is also rarely attained with nanospheres, which also have the disadvantage of a higher polymer content compared with NCs. Finally, these long-circulating PLA-PEG NCs offer several advantages over conventional NC formulations because they can be prepared in total absence of hydrophilic surfactant (poloxamer), are very stable, and may be optimized to obtain higher PEG densities at the surface increasing the plasma circulation time and thus allowing drug delivery to sites outside the MPS.

CONCLUSION

NCs bearing covalent-grafted PEG chains were shown to have modified biodistribution, particularly NCs with a PEG chain length of 20 kDa and 30% PEG content. They remain longer in the bloodstream than NCs with PEG-containing surfactant adsorbed on the surface. These surface-modified NCs will be useful in targeting to endothelial cells and circulating cells, as in the case in some blood parasitic infections, e.g., malaria (29). Extra-vascular sites could be also attained for cancer therapy (30). Other non-parenteral sites of administration, such as the subcutaneous, nasal or oral routes, could also be interesting to investigate.

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