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DNA Nanocarriers for Systemic Administration: Characterization and *In Vivo* Bioimaging in Healthy Mice

Stephanie David[1–3], Catherine Passirani[1,2], Nathalie Carmoy[4,5], Marie Morille[1,2], Mathieu Mevel[3], Benoit Chatin[3], Jean-Pierre Benoit[1,2], Tristan Montier[4–6] and Bruno Pitard[3,7]

We hereby present different DNA nanocarriers consisting of new multimodular systems (MMS), containing the cationic lipid dioleylaminesuccinylparomomycin (DNA MMS DOSP), or bis (guanidinium)-tren-cholesterol (DNA MMS BGTC), and DNA lipid nanocapsules (DNA LNCs). Active targeting of the asialoglycoprotein receptor (ASGP-R) using galactose as a ligand for DNA MMS (GAL DNA MMS) and passive targeting using a polyethylene glycol coating for DNA LNCs (PEG DNA LNCs) should improve the properties of these DNA nanocarriers. All systems were characterized *via* physicochemical methods and the DNA payload of DNA LNCs was quantified for the first time. Afterwards, their biodistribution in healthy mice was analyzed after encapsulation of a fluorescent dye *via in vivo* biofluorescence imaging (BFI), revealing various distribution profiles depending on the cationic lipid used and their surface characteristics. Furthermore, the two vectors with the best prolonged circulation profile were administered twice in healthy mice revealing that the new DNA MMS DOSP vectors showed no toxicity and the same distribution profile for both injections, contrary to PEG DNA LNCs which showed a rapid clearance after the second injection, certainly due to the accelerated blood clearance phenomenon.

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Introduction

Gene therapy is an emerging technology that aims to permanently or temporarily correct a gene defect by the intracellular delivery of nucleic acids.[1] Gene defects can either arise during cell division processes or be due to external agents (such as ultraviolet (UV) or other radiation, chemical substances). The introduction of a plasmid DNA, encoding the native form of the gene can be a way to conquer these gene defects. Since naked plasmid DNA is quickly degraded by blood nucleases and in general shows no relevant therapeutic effects when administered systemically,[1] vectors are necessary to transport plasmid DNA into the cell nucleus. In general, as with any drug, gene transfer complexes must reach their intended site of action to induce therapeutic effects, but this can be compromised through unspecific interactions, especially if they are frequently re-administrated. Consequently, one basic challenge for nonviral gene therapy is to develop an approach that delivers a therapeutic gene into selected cells. With this aim in mind, two different types of promising nanocarriers were developed in our laboratories: lipid nanocapsules (DNA LNCs) and multimodular systems (DNA MMS). DNA LNCs consist of a lipophilic lipid core, containing a mixture of triglycerides and polyglyceryl-6 dioleate surrounded by a shell composed of free polyethylene glycol (PEG) and hydroxystearate-PEG.[2] To encapsulate hydrophilic DNA in the lipophilic lipid core, the first step consists of complexing the anionic DNA with cationic lipids, to form lipoplexes which are then introduced into the formulation process of DNA LNCs, based on phase-inversions of an emulsion.[3] DNA LNCs encapsulating a luciferase-coding plasmid DNA proved their transfection efficacy *in vitro*[4] and, after surface coating with DSPE-PEG_{2000} chains forming PEG DNA LNCs, prolonged their circulation in the blood and their transfection efficacy *in vivo* in a tumor model.[5,6] DNA MMS also exhibit a dual structure: a core composed of lipoplexes and an external corona of steric stabilizers capable of carrying the ligands necessary for active targeting. The first DNA MMS that were developed consisted of the cholesterol derivate BGTC (bis-guanidinium-tren-cholesterol) as a cationic lipid, the plasmid pCMV luciferase, and the steric stabilizer F108 (a block copolymer of poly(ethylene oxide) and poly(propylene oxide)), with or without galactose (GAL) as a ligand. They had already been tested *in vitro* on primary hepatocytes[7] and demonstrated a specific transfection for galactosylated DNA MMS due to the recognition of the GAL ligands by ASGP-R, present on hepatocytes.

With the aim of developing new, efficient nonviral DNA nanocarriers, new MMS containing the aminoglycoside derivate DOSP (dioleylsuccinylparomomycin) were developed

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and compared with the already *in vitro*-tested DNA MMS BGTC. In parallel, the DNA payload in DNA LNCs was for the first time quantified and localized. Afterwards, both DNA MMS, in the presence and absence of GAL, as well as DNA LNCs and PEG DNA LNCs, were injected for the first time *via* intravenous injection into healthy mice and their different biodistribution and kinetics were explored, *via* noninvasive *in vivo* biofluorescence imaging (BFI).[8] Finally, PEG DNA LNCs and DNA MMS DOSP, the DNA nanocarriers with the longest circulation time, were tested for repeated administration because in future therapeutic applications multiple injections can be envisaged to improve the effect of the plasmid DNA.

Results

DNA nanocarrier characterization

DNA MMS DOSP—new MMS for systemic administration. Similar to DNA MMS BGTC, new DNA MMS containing the cationic lipid DOSP were developed. They can be recovered with GAL in order to target the ASGP-R in future applications directed to the liver. To determine their colloidal stability,[9] electrophoresis experiments (**Figure 1a**), size measurements (**Figure 1b**), and fluorescence measurements (data not shown) of DOSP/DOPE/DNA lipoplexes were performed at different charge ratios (CR), defined as the ratio of the cationic lipid charge to the anionic nucleic acid charge (+/–).[10] Three zones of colloidal stability A (CR <2), B (CR 2–5), and C (CR >5) were determined with a complete complexation of the nucleic acids at CR ≥2 indicated by low fluorescence intensities in electrophoresis and fluorescence measurements (data not shown). As a consequence, CR 2 was chosen, but lipoplexes at this CR rate had an increased size and were colloidally unstable. To prevent their aggregation due to their neutral surface charge, the polymer F108 was added to efficiently reduce their size (final size about 100–130 nm, **Figure 1c**). In addition, for all F108/DNA ratios tested, no fluorescence signal was detected in electrophoresis experiments thus indicating no dissociation of the lipoplexes (**Figure 1d**). In view of these results, and in accordance with DNA MMS BGTC, the F108/DNA and F108-gal/DNA ratio of 300 (wt/wt) was chosen.

DNA LNCs—quantification of the DNA payload. To check DNA complexation (lipoplexes) or encapsulation (DNA LNCs) and to confirm that the purification and that the post-insertion process did not modify the DNA encapsulation in PEG DNA LNCs, gel electrophoresis experiments were performed. When LNCs were intact, only very low fluorescence signals were detected for both types of LNCs, indicating neither a loss of DNA nor the liberation of DNA from the DNA LNCs (**Figure 2a**). In contrast, after LNC destruction with Triton, an intense fluorescence signal was observed in both cases. Afterwards, the DNA localization (lipoplexes versus LNCs) was determined using a newly developed quantitative method based on chloroform extraction and subsequent UV spectroscopy analysis at 260 nm. Our hypothesis is that DNA could be potentially localized in four different compartments of the formulation (**Figure 2b**): (1) free DNA molecules, (2) DNA molecules in lipoplexes outside LNCs, (3) lipoplexes encapsulated in LNCs, and (4) DNA molecules encapsulated in LNCs dissociated from cationic lipids. DNA quantification, before purification or post-insertion (**Table 1**), revealed about 16% of free DNA molecules, corresponding to a small line of fluorescence at gel electrophoresis experiments, about 65% in lipoplexes outside DNA LNCs and up to 22% of DNA encapsulated in LNCs, whereas the major part was dissociated from cationic lipids (21 versus 1%). The experimental DNA payload was about 0.07% compared with the theoretical

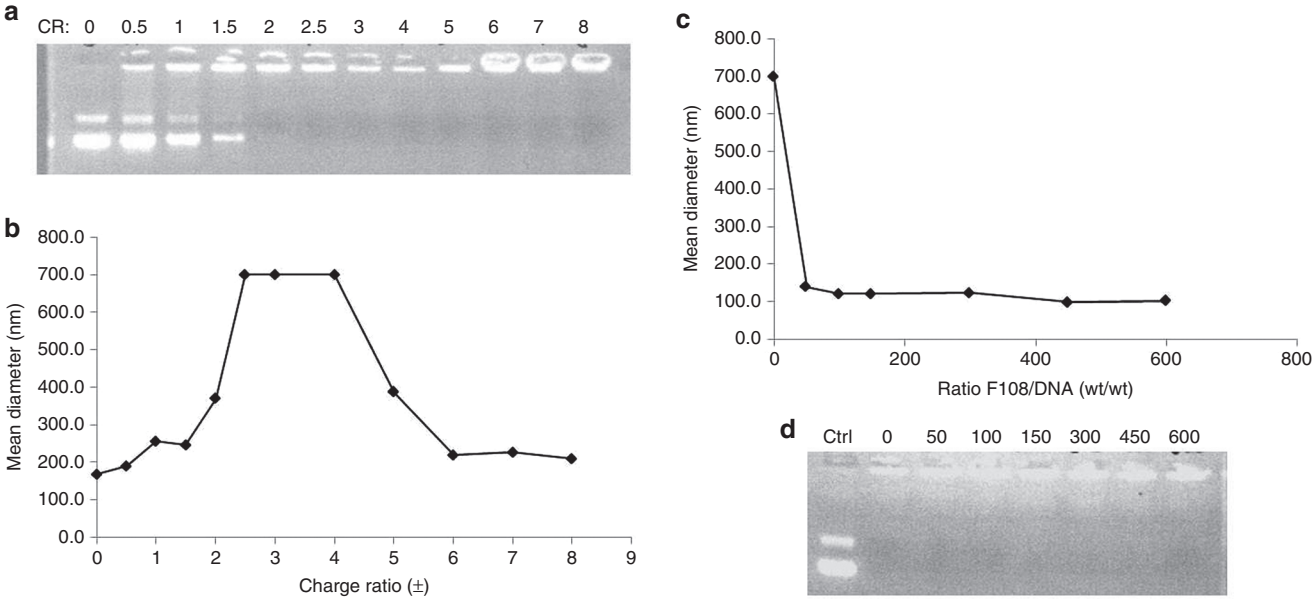

Figure 1 Development of DNA MMS DOSP. (**a**) Electrophoresis experiments and (**b**) size measurements were performed at different charge ratios (CR) of DOSP/DOPE/DNA lipoplexes to determine their colloidal stability. (**c**) Size measurements and (**d**) electrophoresis experiments of DOSP/DOPE/DNA lipoplexes at CR 2 with different polymer/DNA ratios were performed to determine the quantity of polymer used for DNA MMS DOSP. Ctrl, control; DOPE, 1,2-dioleyl-sn-glycero-3-phosphoethanolamine; DOSP, dioleylsuccinylparomomycin; MMS, multimodular system.

DNA payload of 0.29%. To eliminate free DNA, DNA LNCs were purified by gel chromatography before being coated with PEG and used for *in vivo* experiments. Due to the similar size between DNA LNCs and DNA lipoplexes, we hypothesized that these last particles were not totally eliminated by chromatography columns. Consequently, DNA LNCs or PEG DNA LNCs used for the different experiments likely corresponded to a mixture of DNA lipoplexes and LNC. To ensure the homogen composition from batch to batch (size, zeta potential, and DNA payload), all preparations were characterized by physicochemical methods before use for further experiments.

Physicochemical characterization of the different DNA nanocarriers. The lipoplex composition used for DNA LNCs, PEG DNA LNCs, DNA MMS BGTC, GAL DNA MMS BGTC and the newly developed DNA MMS DOSP and GAL DNA MMS DOSP, as well as the polymer/DNA ratios used for *in vivo* experiments are listed in **Table 2**. The plasmid luciferase was initially chosen as a model because it can be quantified quite easily. Before using these DNA nanocarriers for *in vivo* experiments, they were characterized by size and zeta potential measurements (**Table 2**). DNA LNCs had a size of 114 nm with a polydispersity index of 0.3 and a positive zeta potential of +27 mV. Coating the surface with DSPE-PEG_{2000} resulted in a size of 132 nm and a negative zeta potential of –17 mV due to dipolar interactions of PEG with water as previously described.[11] DNA MMS showed sizes of 150 nm (BGTC) and 200 nm (DOSP) with a polydispersity index of 0.4. The addition of GAL resulted in a size of 300 nm for GAL DNA MMS BGTC, which agrees well with previous results,[7] and resulted in a size of 150 nm for GAL DNA MMS DOSP with a polydispersity index of 0.5 and unchanged neutral zeta potentials.

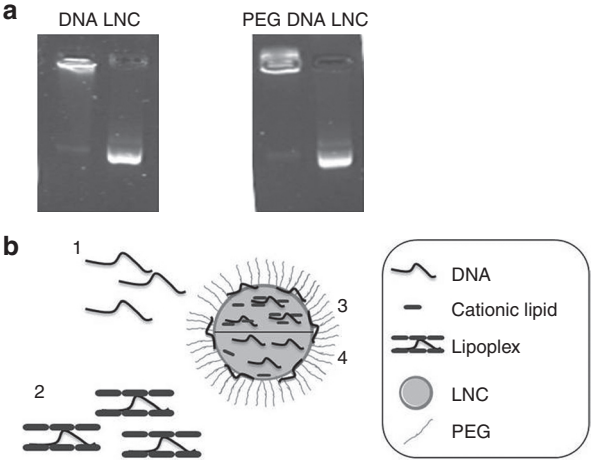

Figure 2 DNA quantification in DNA LNCs. (**a**) Electrophoresis experiments were performed to check DNA encapsulation in DNA LNCs and PEG DNA LNCs. (**b**) Schematic representation of DNA localization in the DNA LNC formulation (1) as free DNA, (2) in lipoplexes outside LNCs, (3) in lipoplexes inside LNCs or (4) in LNCs dissociated from cationic lipids. LNC, lipid nanocapsule; PEG, polyethylene glycol.

In vivo BFI in healthy animals

Biodistribution after one systemic administration. For *in vivo* experiments, nude mice were chosen to avoid hair autofluorescence, to facilitate observations by *in vivo* BFI,[12] and to compare the results with those previously obtained on different tumor models.[5,6,13] To follow the DNA nanocarriers *via* BFI, we used as in a previously reported study[5] the fluorescent dye, DiD (a near-infrared fluorophore used to avoid the autofluorescence wave length emitted by animals) encapsulated in the different DNA nanocarriers and images were taken 1, 3, 5, and 24 hours after systemic administration (**Figure 3**) from lateral and decubitus dorsal views. Different regions of interest were selected to quantify the fluorescence intensities: (1) the abdominal region, including liver and bladder, (2) bladder, (3) liver, and (4) lungs (**Figure 4**). The DNA MMS BGTC and GAL DNA MMS BGTC were rapidly accumulated in the liver. This accumulation persisted during the whole observation period and was similar for both DNA nanocarriers. From the raw data (data not shown), the calculated fluorescence intensities in the liver were three to four times higher than in the bladder and about two times higher than in the lungs. DNA MMS DOSP showed an accumulation in the liver, but less important than for DNA MMS BGTC. A fluorescence signal was also observed in the urinary system, and in the lungs during the whole period with similar intensities than in liver and bladder. Interestingly, DNA MMS DOSP had an increased circulation time compared with the other DNA MMS tested, with fluorescence signals four to five times higher than for GAL DNA MMS DOSP and an increase at 24 hours. The addition of GAL ligands to these systems provided an accentuated accumulation in the liver with less accumulation in the urinary system and the lungs than for DNA MMS DOSP. The fluorescence intensities in the liver were two times higher than in the bladder and about one-third than that of the lungs. DNA LNCs showed a wide degree of distribution 1 hour after intravenous administration, with fluorescence intensities in the liver and the lungs about two times higher than in the bladder. Afterwards, a rapid and important decrease of the fluorescence signal was observed as the signal was divided by two at 3 hours compared with 1 hour. The addition of long

Table 1 DNA quantification in three different formulations before purification and post-insertion with DSPE-PEG

	(1) Free DNA (%)	(2) Free lipoplexes (%)	(3) Encapsulated lipoplexes (%)	(4) Encapsulated DNA (%)	DNA in LNCs (%) (3 + 4)	DNA payload[a] (%)
Formulation 1	0	85 ± 4	1 ± 1	15 ± 6	16 ± 4	0.05 ± 0.01
Formulation 2	21 ± 20	56 ± 34	6 ± 8	21 ± 21	27 ± 14	0.09 ± 0.06
Formulation 3	27 ± 5	53 ± 1	0	21 ± 4	21 ± 4	0.07 ± 0.02
Mean	16 ± 14	65 ± 18	1 ± 2	21 ± 5	22 ± 5	0.07 ± 0.02

LNC, lipid nanocapsule; PEG, polyethylene glycol.
[a]Experimental DNA payload (%) calculated as wt (DNA in LNCs)/wt (formulation) × 100.

Table 2 Characteristics of the different DNA nanocarriers encapsulating the plasmid pGWIZ-luciferase

	DNA nanocarrier specifications					
	DNA LNCs	**PEG DNA LNCs**	**DNA MMS BGTC**	**GAL DNA MMS BGTC**	**DNA MMS DOSP**	**GAL DNA MMS DOSP**
Lipoplex composition						
Lipids	DOTAP/DOPE	DOTAP/DOPE	BGTC/DOPE	BGTC/DOPE	DOSP/DOPE	DOSP/DOPE
Charge ratio	5	5	2	2	2	2
Polymer composition						
Polymer	—	$DSPE\text{-}PEG_{2000}$	F108	F108-gal	F108	F108-gal
Ratio polymer/DNA (wt/wt)	—	70	300	300	300	300
Size (nm)	114 ± 25	132 ± 3	150 ± 32	298 ± 171	198 ± 57	152 ± 58
PDI	0.3	0.3	0.4	0.5	0.4	0.5
Zeta potential (mV)	27 ± 12	−17 ± 4	−3 ± 2	−2 ± 1	0	−2 ± 0

BGTC, bis (guanidinium)-tren-cholesterol; DOPE, 1,2-dioleyl-sn-glycero-3-phosphoethanolamine; DOSP, dioleylsuccinylparomomycin; DOTAP, 1,2-dioleyl-3-trimethylammoniumpropane; GAL, galactose; LNC, lipid nanocapsule; MMS, multimodular system; PDI, polydispersity index; PEG, polyethylene glycol.

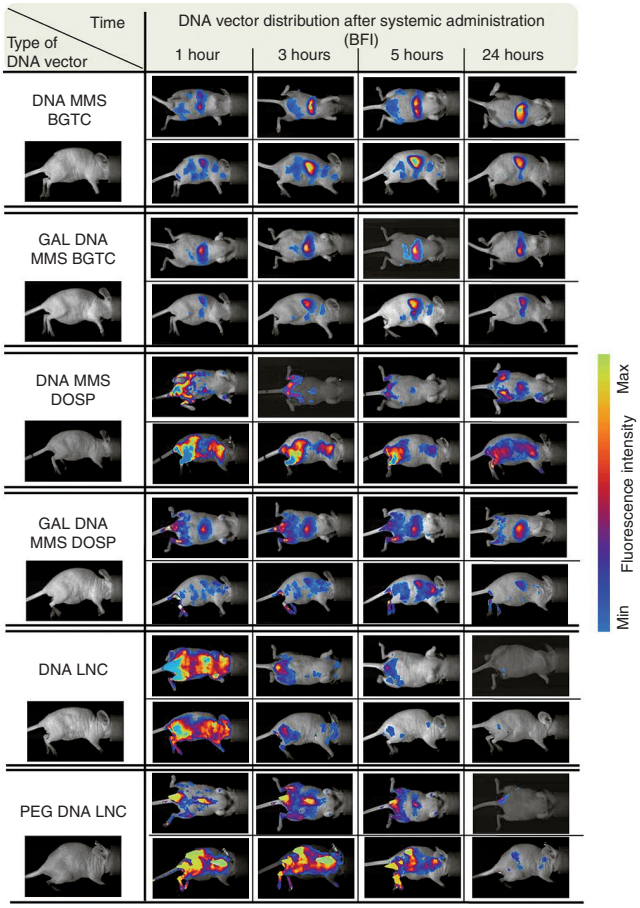

Figure 3 BFI of the different DNA nanocarriers administered via intravenous injection in healthy animals at different times. DNA MMS BGTC, GAL DNA MMS BGTC, DNA MMS DOSP, GAL DNA MMS DOSP, DNA LNCs, and PEG DNA LNCs encapsulating a fluorescent tracer, DiD, were injected intravenously in healthy mice (two animals per group) and BFI images were taken at different times (1, 3, 5, and 24 hours after injection) from decubitus dorsal (first line) and lateral (second line) views to follow their biodistribution. BFI, biofluorescence imaging; BGTC, bis (guanidinium)-tren-cholesterol; DOSP, dioleylsuccinylparomomycin; GAL, galactose; LNC, lipid nanocapsule; MMS, multimodular system; PEG, polyethylene glycol.

DSPE-PEG chains augmented the circulation time up to 5 hours after administration with a maximal signal at 3 hours and showed a wide distribution in the whole body as with DNA LNCs at 1 hour. However, 24 hours after their administration, no fluorescence was detected on the images as the fluorescence signal was only about 60% of the initial signal, indicating the elimination of these systems.

Biodistribution after repeated administration. As the repeated administration of these systems could be envisaged in a treatment context (chronic disease), the two systems with the longest circulation profile (PEG DNA LNCs and DNA MMS DOSP) were followed *via* BFI after two intravenous injections administered at time intervals of 1 week (**Figures 4d** and **5**). Images of the first injection have already been described in the previous section. After the second injection of DNA MMS DOSP, the biodistribution profile was similar to the first one, represented by a prolonged circulation time and an increase of the fluorescence signal at 24 hours. However, the fluorescence signals in the liver and in the lungs were, that time, twice higher than in the bladder, like for DNA LNCs. The biodistribution profile for the PEG DNA LNCs after the second injection showed a reduced circulation time and a similar profile to DNA LNCs, with wide distribution in the whole body 1 hour after administration. In contrast, an augmentation of the signal in the lungs and the liver to 2.5 times than that in the bladder after the second injection and a high level of intolerance to PEG DNA LNCs after the second injection were observed.

Hepatotoxicity of PEG DNA LNCs and DNA MMS DOSP. To determine the hepatotoxicity of PEG DNA LNCs and DNA MMS DOSP, blood samples were collected regularly during the observation period and the enzyme activity of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were quantified (**Figure 6**) indicating a hepatotoxicity when both ALAT values, specific to the liver, and ASAT values, also found in other organs and tissues, were increased. For the group receiving PEG DNA LNCs, ASAT and ALAT values showed a slight increase 24 hours after the first injection. These values increased again, but to a lesser extent, 24 hours after the second injection. However, these values are near to the values obtained for the control group

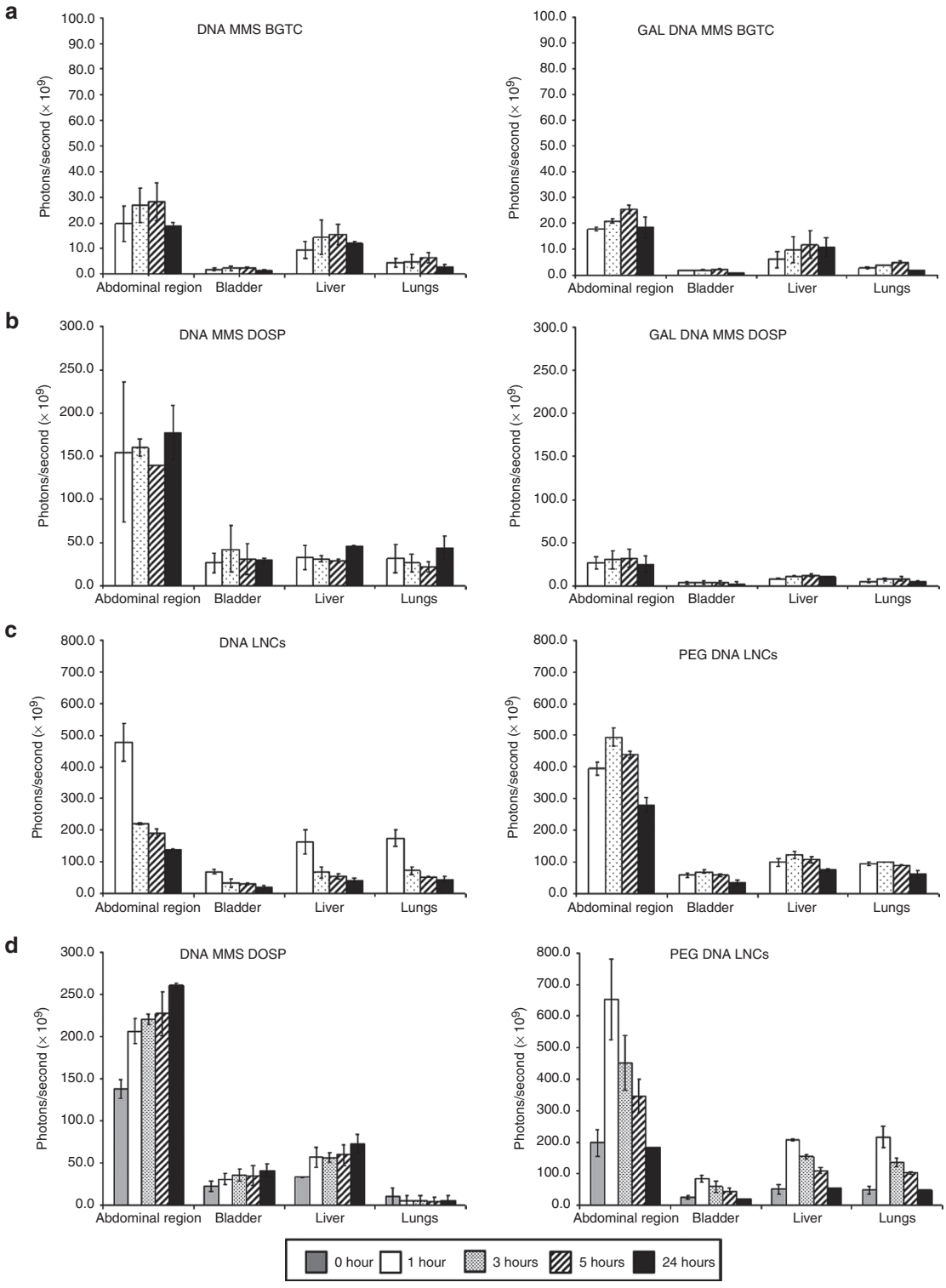

Figure 4 Fluorescence quantification after systemic administration of the different DNA nanocarriers in healthy mice. Different regions of interest were selected to quantify the fluorescence signal from images taken *via* BFI (**Figures 3** and **5**) at different times (1, 3, 5, and 24 hours after the first injection, or 0 (before), 1, 3, 5, and 24 hours after the second injection) (two animals per group): (1) the abdominal region, including liver and bladder, (2) bladder, (3) liver, and (4) lungs. Values were normalized with the values of control animals (receiving no injection). (**a**) Animals receiving one injection of DNA MMS BGTC or GAL DNA MMS BGTC, (**b**) animals after one injection of DNA MMS DOSP or GAL DNA MMS DOSP, (**c**) animals after one injection of DNA LNCs or PEG DNA LNCs, and (**d**) animals after two injections of DNA MMS DOSP or PEG DNA LNCs. BFI, biofluorescence imaging; BGTC, bis (guanidinium)-tren-cholesterol; DOSP, dioleylsuccinylparomomycin; GAL, galactose; LNC, lipid nanocapsule; MMS, multimodular system; PEG, polyethylene glycol.

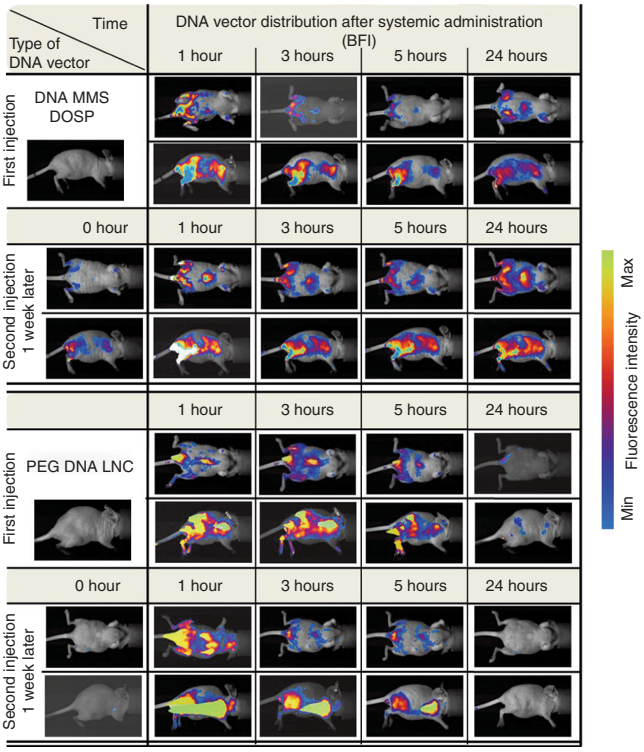

Figure 5 BFI of DNA nanocarriers in healthy animals after repeated, systemic administration. PEG DNA LNCs and DNA MMS DOSP encapsulating the fluorescent dye DiD were injected twice intravenously in healthy animals with a time interval of 1 week between administrations and BFI images were taken (from two animals per group) at different times (1, 3, 5, and 24 hours after each injection and 1 day before the second injection (0 hour)) from decubitus dorsal (first line) and lateral (second line) views to follow their biodistribution. BFI, biofluorescence imaging; DOSP, dioleylsuccinylparomomycin; LNC, lipid nanocapsule; MMS, multimodular system; PEG, polyethylene glycol.

(without nanocarrier injection). ASAT and ALAT values for the mice receiving DNA MMS DOSP, showed nearly the same profile as the ASAT and ALAT values for the control group indicating no hepatotoxicity of these DNA MMS.

Fluorescence accumulation and luciferase quantification in different organs 24 hours after intravenous injection. Twenty-four hours after the last administration of DNA nanocarriers (two administrations for PEG DNA LNCs and DNA MMS DOSP; one administration for DNA LNCs, DNA MMS BGTC, GAL DNA MMS BGTC, and GAL DNA MMS DOSP; and no injection for the control group), the animals were killed and the fluorescence localization was determined in different organs (liver, lungs, kidneys, spleen, and heart) to confirm BF images since BFI is affected by tissue depth[8] (**Figure 7**). For all DNA nanocarriers, the major intensity was observed in the liver, followed by some fluorescence in the lungs and almost no fluorescence intensity in the heart, the spleen, and the kidneys. In mice receiving the two injections, the luciferase expression was also quantified but revealed little luciferase expression (<2 relative luciferase unit/mg proteins) (data not shown).

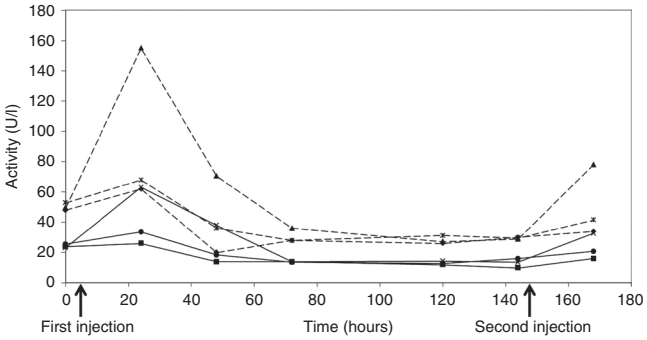

Figure 6 ALAT and ASAT quantification to determine hepatotoxicity of PEG DNA LNCs and DNA MMS DOSP. Blood samples of animals receiving no injection (control, closed diamonds), PEG DNA LNCs (closed triangles) or DNA MMS DOSP (closed circles) were collected once a day during the observation period, starting 1 hour before the injection of the DNA nanocarriers, to analyze the hepatotoxicity of these DNA nanocarriers, represented by ALAT (continuous lines) and ASAT values (dashed lines) (n = 2 for untreated animals and n = 5 for treated animals). ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; DOSP, dioleylsuccinylparomomycin; LNC, lipid nanocapsule; MMS, multimodular system; PEG, polyethylene glycol.

Discussion

New MMS containing the lipids DOSP/DOPE (1,2-dioleyl-sn-glycero-3-phosphoethanolamine) were developed showing comparable physicochemical characteristics as previously described for DNA MMS BGTC. In parallel, DNA was quantified and localized for the first time using a method based on chloroform extraction and spectroscopy analysis and revealed about 22% of DNA being encapsulated in LNCs. The major part of this DNA was dissociated from cationic lipids, certainly due to a rearrangement of the different lipids around the nucleic acids during the formulation process, since lipoplexes with an initial size of about 400 nm were encapsulated in DNA LNCs presenting a size of about 100 nm.[2] However, a large part of the DNA was still complexed in lipoplexes outside DNA LNCs which remained in the formulation even after the purification step, carried out to eliminate the excess of free components, since they both have similar sizes. This was also confirmed by electrophoresis analysis that, showed similar fluorescence intensities for DNA LNCs (before the purification step) and PEG DNA LNCs (after the purification step) (**Figure 2a**). Most of these (PEG) DNA LNCs and DNA MMS, developed in our laboratories, presented appropriate characteristics for systemic administration.

In vivo BFI, a fast, simple, and relatively low-cost technique,[14] was used to determine the biodistribution profiles of the DNA nanocarriers in healthy mice. With this aim in mind, the fluorescent tracer, DiD, which is not soluble in water and is known to be prone to aggregation and auto-quenching in aqueous buffer,[15] was encapsulated in DNA nanocarriers, which mimicked an "organic-like" medium, and thus improved its optical properties. However, the fluorescence intensity is not an absolutely quantitative method and depends on the tissue observed and its localization.[16] Furthermore, the quantity of DiD was not equal in all systems, but quantities in DNA MMS with the different lipids and ligands were equal, so they could

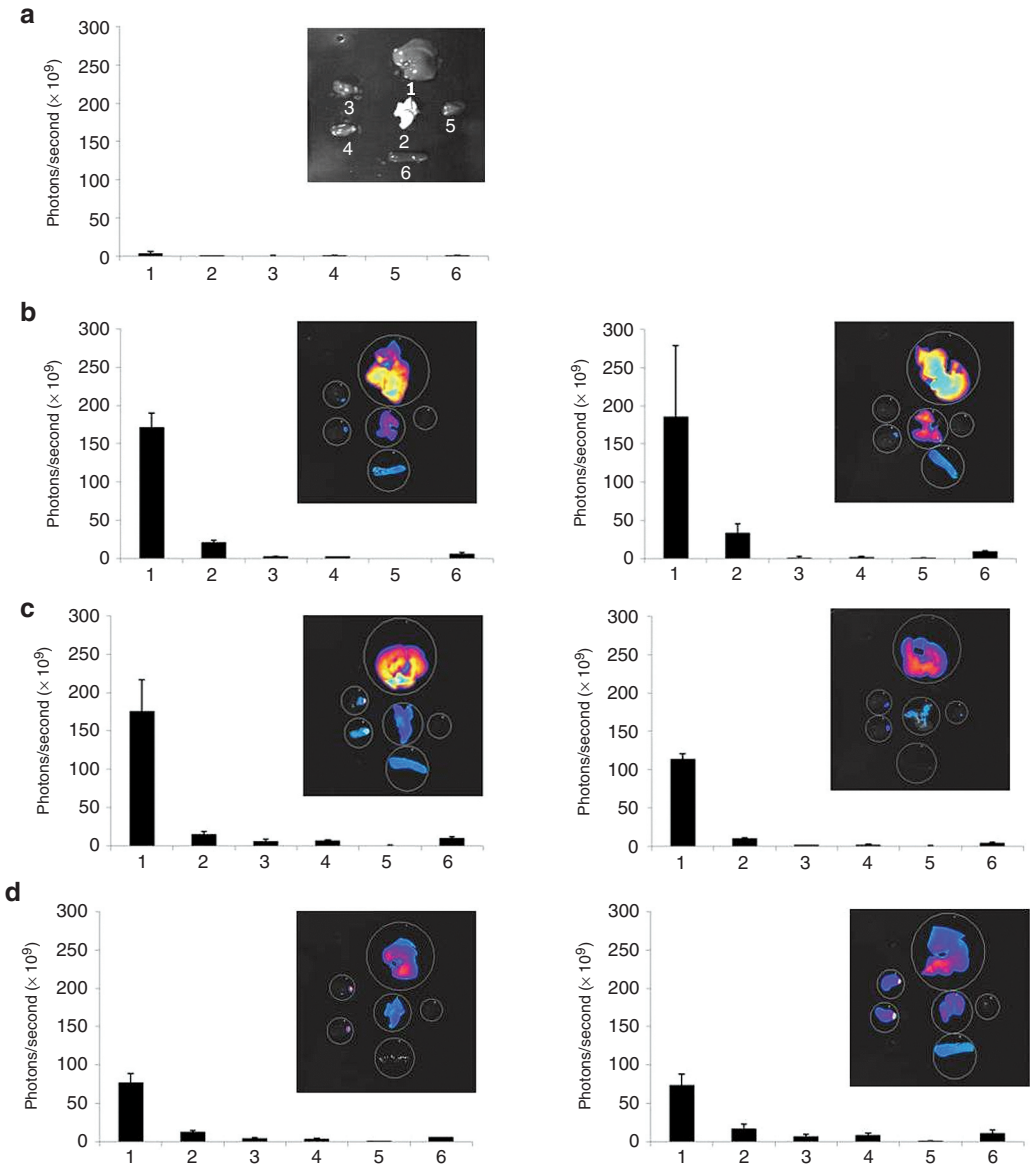

Figure 7 BFI of DNA nanocarriers in different organs 24 hours after the last systemic administration. Animals were killed 24 hours after the last injection of DNA nanocarriers and the fluorescence intensity in (1) liver, (2) lungs, (3 and 4) kidneys, (5) heart, and (6) spleen were measured *via* BFI. All images were taken with the same settings. (**a**) Control animals receiving no injection ($n = 2$), (**b**) animals receiving one DNA MMS BGTC injection ($n = 2$) (left) or one GAL DNA MMS BGTC injection ($n = 2$) (right), (**c**) animals receiving two DNA MMS DOSP injections ($n = 3$) (left) or one GAL DNA MMS DOSP injection ($n = 2$) (right), (**d**) animals receiving one DNA LNC injection ($n = 2$) (left) or two PEG DNA LNC injections ($n = 4$) (right). BFI, biofluorescence imaging; BGTC, bis (guanidinium)-tren-cholesterol; DOSP, dioleylsuccinylparomomycin; GAL, galactose; LNC, lipid nanocapsule; MMS, multimodular system; PEG, polyethylene glycol.

be compared on the one side, and quantities in DNA LNCs and PEG DNA LNCs on the other side, were also equal and could thus also be compared with each other. Each DNA nanocarrier showed a specific biodistribution profile in function of its composition. DNA MMS composed of the cationic lipid BGTC had a short circulation time and accumulated preferentially in the liver whereas DNA MMS composed of the cationic lipid DOSP favored prolonged circulation time, as evidenced by a clear visible accumulation up to 24 hours after the first injection. The addition of the ligand GAL to both systems resulted in an accumulation in the liver. This phenomenon is certainly due to the ASGP-R situated on hepatocytes which recognizes terminal, GAL-bearing ASGP and favors the specific internalization of GAL-containing systems.[7,17,18] DNA LNCs showed wide distribution but a short circulation time which could be increased by being coated with DSPE-PEG chains, as has also been seen previously on different tumor models.[5,6,13]

Afterwards, the DNA MMS DOSP and PEG DNA LNC systems which had the longest circulation times were chosen for repeated administration on healthy animals. DNA MMS DOSP showed no hepatotoxicity and similar biodistribution profiles for both injections. Interestingly, the fluorescence signal of these systems augmented, contrary to than of the other systems, at 24 hours for both injections. This could be due to

a re-distribution or re-metabolization of the labeled lipid, but as it was only observed with these systems, it is likely that this phenomenon could be attributed to the DNA complexes rather than the tracer itself. In contrast, PEG DNA LNCs were rapidly eliminated and showed an augmented intolerance on healthy mice after the second injection. The elimination process is probably due by the mononuclear phagocyte system,[5] as these DNA nanocarriers are too big for renal elimination that is extremely size restricted.[19] The fluorescence signal observed in the bladder is likely due to the biodegradation of the systems occurring inevitably with time after their injection. Mice presented signs of paralysis at their extremities and difficulty in breathing, typical of a shock response, to the point of rapid mortality within 10–30 minutes after the injection of some animals. This could be due to the accelerated blood clearance phenomenon of the PEGylated carriers in combination with their inflammatory payload (plasmid DNA) and the use of athymic (nude) mice. This phenomenon has already been reported in the literature[20–22] but is still not fully elucidated. Two distinct phases can be determined in this phenomenon.[23,24] (i) The induction phase following the first injection, where liposomes, or in our case PEG DNA LNCs, bind and cross-link surface immunoglobulin on PEG-reactive B-cells,[25] inducing the production of anti-PEG IgM and (ii) the effectuation phase following the second injection, whereas on the one hand, the DNA payload is internalized followed by B-cell stimulatory pathway activation, such as TLR 9[26] and on the other hand, accessory cells are induced to produce cytokines, independent of T helper cell[26–28] followed by the rapid clearance of the PEGylated carriers from the bloodstream, mainly by Kupffer cells in the liver. The severe reactions observed here, including the death of some mice, could be due to the non-regulation of the immune response due to the use of nude (athymic) mice,[22,29,30] but the regulation is complex and needs further investigations to be fully understood.

For the long-circulating systems, PEG DNA LNCs and DNA MMS DOSP, no luciferase or very low expression could be detected 24 hours after the last injection in any organ although fluorescence was observed in the liver. This is in good agreement with our object whether to transfect only deficient cells and no healthy cells. To obtain transfection, a targeting strategy is necessary as was observed in other *in vivo* studies with grafted tumors after intravenous injection of PEG DNA LNCs (passive targeting)[6] and GAL DNA MMS DOSP (active targeting).[31]

The absence of transfection in the physiological liver, observed here, could suggest that the number of hepatocytes transfected by nanoparticles compared with the total number of hepatocytes is not sufficient to produce enough luciferase to be detected. In contrast, by using targeting strategies, as was done in the other studies, accumulated doses were probably greater which led to more efficient transfection. For PEG DNA LNCs, an important point for the absence of luciferase expression is certainly the rapid clearance of these DNA nanocarriers after the second injection due to the accelerated blood clearance phenomenon. For the future, there are different possibilities to diminish this immunogenic response by considering the time interval between the different injections,[21,32] the lipid composition of the PEG component,[21,33] the sequence of the pDNA,[22] and/or the animals[20] used.

In summary, we have presented here various DNA nanocarriers for systemic administration with appropriate physicochemical properties and different biodistribution profiles depending on their lipid and surface composition. These DNA nanocarriers represent a promising tool for various applications such as tumor targeting or hepatocyte targeting. Furthermore, this platform can easily be complemented using other lipids and/or ligands.

Materials and Methods

DNA nanocarrier preparations. All DNA nanocarriers used were based on lipoplex formation prepared by adding equal volumes of DNA plasmid (pgWIZ-luciferase (Gene Therapy systems, San Diego, CA)) and liposomes in a defined CR of cationic lipid charge and anionic DNA charge to obtain a final DNA concentration of 0.25 g/l for DNA MMS or 0.825 g/l for DNA LNCs. NaCl was added during preparation, to obtain a final concentration of 0.15 mol/l. Lipoplexes were incubated for 20 minutes at room temperature before use. For liposome preparation, a cationic lipid DOSP (synthesis previously described in ref. [34]), BGTC (synthesis previously described in ref. [35]) or DOTAP (1,2-dioleyl-3-trimethylammoniumpropane) (Avanti Polar Lipids, Alabaster, AL) was weighted with the neutral lipid DOPE (Avanti Polar Lipids) at the ratios 1/1, 3/2, and 1/1 (mol/mol), respectively to obtain a final concentration of 20 mmol/l of cationic lipid charge (considering the number of positive charges per molecule: 4 for DOSP, 2 for BGTC, and 1 for DOTAP), and solubilized in chloroform. Chloroform was then evaporated under vacuum to obtain a homogeneous lipid film which was hydrated with deionized water overnight at 4 °C. The next day, liposomes were sonicated and size measurement was performed before use. To prepare DNA MMS for BFI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD, emission = 644 nm; excitation = 665 nm) (Invitrogen, Cergy-Pontoise, France) was added to the lipids before lipid film preparation and the steric stabilizers F108 (80% poly(ethylene oxide), molecular weight 14,600, generously provided by BASF, Ludwigshafen, Germany) or F108-gal (synthesis previously described in ref. [7]) were added to DOSP/DOPE or BGTC/DOPE liposomes before lipoplex preparation. To obtain fluorescent DNA LNCs for BFI, DOTAP/DOPE/DNA lipoplexes (CR = 5), corresponding to 78.9% (wt/wt), were added to 9.9% (wt/wt) lipophilic Labrafac WL 1349 (Gatefossé S.A., Saint-Priest, France) mixed with DiD as described in ref. [36], 3.9% (wt/wt) oleic Plurol (Polyglyceryl-6 dioleate) which was kindly provided by Gatefossé S.A., 1.4% (wt/wt) NaCl (Prolabo, Fontenay-sous-Bois, France), and 5.9% (wt/wt) Solutol HS-15 (BASF).[3] Briefly, after mixing all the components, temperature cycles around the phase-inversion temperature were performed under magnetic stirring. Later, ice-cooled water (obtained from a Milli-Q-plus system; Millipore, Paris, France) was added (at a ratio of 1:1.96) to dilute the obtained microemulsion and form LNCs. To eliminate free components, DNA LNCs were purified, using PD10 Sephadex columns (Amersham Biosciences Europe, Orsay, France), ultrafiltrated with MilliporeAmicon Ultra-15 centrifugal filter devices (Millipore, St Quentin-Yvelines, France) and then the salt and LNC concentrations were readjusted to obtain a physiologic concentration of NaCl (150 mmol/l) and the initial concentration of LNCs (152 g/l).[2] PEG

DNA LNCs with a final polymer concentration of 10 mmol/l were obtained by a post-insertion process which consisted of mixing purified DNA LNCs with DSPE-mPEG$_{2000}$ (1,2-DiStearoyl-sn-glycero-3-PhosphoEthanolamine-N-[methoxy (polyethylene glycol)-2000], mean molecular weight = 2,805 g/mol; Avanti Polar Lipids), and an incubation for 4 hours at 30 °C by vortexing every 15 minutes.[5]

DNA nanocarrier characterization

Size and zeta potential measurements. Size measurements for DNA MMS DOSP development described in the first section of the results were performed using a Malvern Zetasizer 300HSA (Malvern Instruments S.A., Worcestershire, UK) with a dilution of 4:100 in 0.15 mol/l NaCl. Size and zeta potential measurements for DNA nanocarrier characterization were performed using a Malvern Zetasizer (Nano Series ZS; Malvern Instruments S.A.) at 25 °C, in triplicate after dilution at a ratio of 1:100 with deionized water for DNA LNCs or at a ratio of 4:100 with 0.15 mol/l NaCl for DNA MMS DOSP.

Agarose gel electrophoresis. Sample preparation for electrophoresis experiments with the aim of DNA MMS DOSP development, described in the first section of the results, was performed by mixing complexes with Orange Blue loading dye (Promega, Madison, WI). In contrast, a sample preparation for electrophoresis experiments with (PEG) DNA LNC formulations was performed as previously described.[2] Briefly, a treatment with Triton X100 (Sigma, Saint-Quentin Fallavier, France) was performed to destroy a volume of LNCs equivalent to 0.2 µg of DNA and samples with or without treatment were mixed with gel-loading solution (Sigma). In both cases, the prepared samples were then deposited on 1% agarose gel containing ethidium bromide (Sigma) to migrate for about 30 minutes at 100 V.

DNA quantification in DNA LNCs. To analyze the free and encapsulated DNA quantity in DNA LNCs, a volume of DNA LNCs was mixed with four volumes of water (obtained from a Milli-Q-plus system; Millipore) and one volume of chloroform, vortexed and immediately centrifuged for 15 minutes at 12,600 rpm at 4 °C. The aqueous phase, containing free DNA, was removed and analyzed with a UV spectrophotometer (UVIKON 922; Kontron Instruments, Munic, Germany) at 260 nm. The volume removed for quantifying the free DNA was replaced by pure ethanol, to liberate the DNA encapsulated in the DNA LNCs, and six volumes of water were added before vortexing and immediately centrifuging a second time for 15 minutes at 12,600 rpm at 4 °C. The aqueous phase, containing the liberated DNA from DNA LNCs, was removed and analyzed as previously with a UV spectrophotometer (UVIKON 922; Kontron Instruments) at 260 nm. To analyze the DNA quantity complexed with cationic lipids inside or outside the DNA LNCs, the same procedure was used, but water was replaced by 1 mol/l of NaOH to dissociate the lipoplexes. The first aqueous phase contained the DNA liberated from lipoplexes outside DNA LNCs; the second aqueous phase contained the DNA liberated from lipoplexes inside DNA LNCs. The DNA quantity was calculated using a calibrating curve with different DNA concentrations and compared with the total DNA amount encapsulated in theory in DNA LNCs.

In vivo experiments

DNA nanocarrier administration. Six- to nine-weeks-old female, nude SWISS mice (Charles River, L'Arbresle, France) were housed and maintained at the University animal facility; they were processed in accordance with the Laboratory Animal Care Guidelines (NIH Publication 85-23, revised 1985) and with the agreement of the regional veterinary services (authorization FR; 29-024). The different nanocarriers were injected at volumes of 150 µl for DNA LNCs and PEG DNA LNCs, and 200 µl for DNA MMS, by intravenous injection into the tail vein of the mice. Animals receiving PEG DNA LNCs and DNA MMS DOSP were injected twice at a time interval of 1 week between the two injections. The animals were killed 24 hours after the last intravenous injection.

In vivo BFI. To follow the biodistribution of the different nanocarriers, noninvasive fluorescent imaging (BFI) was performed 1, 3, 5, and 24 hours post-injection as described before on two animals per group.[13] Briefly, the BFI system of the NightOWL II (Berthold Technologies, Stuttgart, Germany) equipped with a cooled, slow-scan CCD camera and driven with the WinLight 32 software (Berthold Technologies) was used, using the 590 nm excitation and 655 nm emission filters. Each mouse was anesthetized with isoflurane during the acquisition time (3 seconds for one fluorescent acquisition). The fluorescent signal was then quantified and overlaid on a picture of each mouse.

Fluorescence and luciferase quantification in different organs. Twenty-four hours after the last DNA nanocarrier injection, animals were killed and the heart, lungs, spleen, liver, and kidneys were dissected. Organs from animals receiving no or one DNA nanocarrier injection (two animals per group) and organs from half of the animals receiving two DNA nanocarrier injections (three animals for the PEG DNA LNCs group and four animals for the DNA MMS DOSP group) were immediately placed in the BFI system and biofluorescence images were taken using the same settings as for the whole animals. Organs from the other half of the animals receiving two injections (four animals for the PEG DNA LNCs group and five animals for the DNA MMS DOSP group) were placed in tubes with PLB 1x (Passive Lysis Buffer; Promega France, Lyon, France) and shred with the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) for luciferase quantification. Tubes were centrifuged for 10 minutes at 1,150*g* at 4 °C and the upper phase was transferred into Eppendorf tubes. After another centrifugation of 10 minutes at 20,000*g* at 4 °C, 25 µl of the upper phase was placed, in triplicate, in a white, 96-well plate and the quantification with the luciferin reagent (Promega France) was performed with the MLX luminometer plate reader (Dynex, Guyancourt, France).

ALAT—ASAT determination. Blood samples were collected from the lateral saphenous vein as described in ref. [37] from animals receiving no injections (n = 4), PEG DNA LNCs (n = 9), and DNA MMS DOSP (n = 9). Blood was collected once a day during the analyzing period on different animals (two untreated animals and five treated animals per day) to prevent a too great loss of blood, and were collected in Microvette collection tubes (Sarstedt, Numbrecht, Germany). Afterwards, the samples were centrifuged for 2 minutes at

10,000*g* at 4 °C and the plasma removed for further analysis. ALAT and ASAT values were determined using a Selectra-E (Elitech, Signes, France).

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