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Galactosylated DNA lipid nanocapsules for efficient hepatocyte targeting

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ABSTRACT

The main objective of gene therapy via a systemic pathway is the development of a stable and non-toxic gene vector that can encapsulate and deliver foreign genetic materials into specific cell types with the transfection efficiency of viral vectors. With this objective, DNA complexed with cationic lipids of DOTAP/DOPE was encapsulated into lipid nanocapsules (LNCs) forming nanocarriers (DNA LNCs) with a size suitable for systemic injection (109 ± 6 nm). With the goal of increasing systemic delivery, LNCs were stabilised with long chains of poly(ethylene glycol) (PEG), either from a PEG lipid derivative (DSPE-mPEG$_{2000}$) or from an amphiphilic block copolymer (F108). In order to overcome internalisation difficulties encountered with PEG shield, a specific ligand (galactose) was covalently added at the distal end of the PEG chains, in order to provide active targeting of the asialoglycoprotein-receptor present on hepatocytes. This study showed that DNA LNCs were as efficient as positively charged DOTAP/DOPE lipoplexes for transfection. In primary hepatocytes, when non-galactosylated, the two polymers significantly decreased the transfection, probably by creating a barrier around the DNA LNCs. Interestingly, galactosylated F108 coated DNA LNCs led to a 18-fold increase in luciferase expression compared to non-galactosylated ones.

1. Introduction

In the field of gene delivery, the most common lipid-based carriers of polynucleotides are lipid–DNA complexes, also called lipoplexes, obtained by mixing cationic liposomes with DNA at a precise ± charge ratio. Whereas many cell types are transfected *in vitro* with cationic lipids (thanks to electrostatic interactions between negatively charged membranes and positively charged systems), the excess of cationic lipids used to complex DNA can lead to high cytotoxicity. Dose-dependent toxicity of different kinds of lipoplexes [GL-67 (Lee et al., 1996) (*N*4-spermine cholesterylcarbamate) and GL-62 (Lee et al., 1996) (*N*1-spermine cholesterylcarbamate), DMRIE (1,2-dimyristoyloxypropyl-3-dimethyl-hydroxyethylammonium bromide), DOTMA:DOPE] has been observed, for instance, after their injection into mice leading to hair erection and lethargy (Tousignant et al., 2000). Clinical studies have also shown dose-dependent haematological and serological changes typified by profound leukopaenia, thrombocytopaenia, and elevated serum transaminase levels, indicative of hepatocellular necrosis. Moreover, another obstacle in the use of lipoplexes via systemic delivery is their aggregation, instability and propensity to be captured by the mononuclear phagocyte system (MPS). Indeed, positively charged particles can be opsonised with plasma proteins such as immunoglobulin M, complement C3, and proteins from the coagulation cascade (Ogris et al., 1999) leading to their rapid clearance by MPS phagocyte cells in the liver, spleen, lungs and bone narrow (Brigger et al., 2002).

In this context, numerous efforts have been carried out to obtain effective, stable and long-circulating gene-delivery systems, mostly using the dissimulation of the positive charges by 'shielding' the vector surface with hydrophilic and flexible polymers such as poly(ethylene glycol) (PEG). The use of this surface modification (also called pegylation) of vectors destined for systemic injection has drawn considerable interest (Klibanov et al., 1990; Letrou-Bonneval et al., 2008; Wong et al., 1997).

In parallel to the studies based on lipoplexes, nanoparticle-based systems have been developed. With the aim of use for systemic injection, dissimulating DNA from blood nucleases by encapsulation in nanocapsules seems to be the best alternative to keep the integrity of nucleic acids (Morille et al., 2008). Heurtault et al. (2002) developed lipid nanocapsules synthesized by a solvent-free method and covered by PEG$_{660}$ at a high density, allowing really weak complement activation and low macrophage uptake (Vonarbourg et al., 2006b). In a previous work, the formulation of these nanocapsules was adapted to obtain DNA nanocapsules (DNA LNCs) (Vonarbourg et al., 2009). The lipid core allowed the entrapment of plasmid DNA molecules after the formation of lipoplexes. The DNA LNCs were small (109 ± 6 nm), suitable for intravenous injection, but *in vivo* stability and plasmatic half-life remained low and ill-adapted to efficient *in vivo* transfection. For these reasons

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we chose to modify the surface of this gene-delivery system, inserting longer PEG chains to the surface of DNA LNCs between the already existing dense PEG_{660} chains from the nanoencapsulation process. This was carried out by using two kinds of amphiphilic and flexible polymers. The first one was F108, a block copolymer, consisting of ethyleneoxide (EO) and propyleneoxide (PO) blocks arranged in a triblock structure (EO_{132}–PO_{50}–EO_{132}). The second one was a lipid PEG derivative, the 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-$mPEG_{2000}$). We demonstrated a significant increase of *in vivo* circulation time in mice, especially for the DSPE-$mPEG_{2000}$ coating, with a $t_{1/2}$ of elimination of about 7 h, i.e. around 5-fold more than for non-coated DNA LNCs (submitted results).

Nevertheless, coating vectors with PEG presents some drawbacks: although the PEG coating enhances plasma circulation time and consequently leaves time for the objects to reach their targets (Choi et al., 1998; Kwoh et al., 1999; Ogris et al., 1999), it also represents a major barrier for internalisation and endosomal escape (Erbacher et al., 1999; Song et al., 2002). This is the paradox of PEG use: this polymer has to protect the vector in blood circulation, but once at its target site, the carrier has to uncoat itself to allow its internalisation in the targeted cells, endosomal escape, and finally transport into the nuclei (Morille et al., 2008). Therefore, a compromise has to be found between sufficient circulation time in the blood and efficient transfection. A solution to overcome these difficulties and to avoid potential problems of non-specific interactions, is to attach a specific ligand to the gene-delivery system, resulting in active targeting and receptor-mediated endocytosis (Moffatt et al., 2005; Ogris et al., 2003; Xu et al., 2002). Among the cellular targets for such ligands, the asialoglycoprotein-receptor (ASGP-R) is expressed exclusively on hepatocytes (Stockert, 1995): ASPG-R naturally binds and internalises the terminal galactose-binding asialoglycoprotein. Thus, galactose molecules grafted at the surface of a carrier could provide an active targeting. This can be useful for efficient targeting of the liver, and a consequent secretion of therapeutic gene products into systemic circulation.

We first chose in this study to test the influence of F108 and DSPE-$mPEG_{2000}$ on the transfection efficiency of DNA LNC on cancer cell lines. In a second time, the two kinds of polymers used for the coating were galactosylated (DSPE-PEG-gal and F108-gal), following different synthesis pathways, and the so formed galactosylated DNA LNCs were tested for their *in vitro* transfection efficiency on primary hepatocytes, with the goal to evidence a specific transfection of these vectors.

2. Materials and methods

2.1. Preparation of carriers

2.1.1. Liposome/lipoplexe preparation

DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and DOPE (1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine) (Avanti Polar Lipids, Inc., Alabaster, USA) were first dissolved in chloroform (Sigma, Saint-Quentin Fallavier, France) and then dried by evaporation under vacuum. The formed lipid film was hydrated with deionized water. Then liposomes were sonicated in a bath for 20 min. Lipoplexes were prepared by mixing DOTAP/DOPE (1/1, M/M) liposomes at a positive charge concentration of 25 mM with 660 μg of luciferase encoding plasmid (Pitard et al., 2002) (gWIZ-luc, 6732 bps, amplified and purified for research grade, by GENEART, Regensburg, Germany) at a charge ratio (±) of 5 per 150 mM NaCl.

2.1.2. DNA-loaded lipid nanocapsules (DNA LNCs)

The formulation of LNCs was based on a phase-inversion process described by Heurtault et al. (2003). LNCs were made with lipophilic Labrafac® WL 1349 (caprylic-capric acid triglycerides, European Pharmacopia, IVth, 2002) and oleic Plurol® (Polyglyceryl-6 dioleate) which were kindly provided by Gattefossé S.A. (Saint-Priest, France) and Solutol® HS-15 (70% of PEG 660 hydroxystearate (HS-PEG) and 30% of free PEG 660 Dalton (European Pharmacopia, IVth, 2002) which was a gift from BASF (Ludwigshafen, Germany). Briefly, 3.9% of oleic Plurol® (w/w), 5.9% of Solutol® (w/w), 9.9% of Labrafac® (w/w), 78.9% of water (w/w) and 1.4% of NaCl, were mixed together under magnetic stirring. Previously formed DOTAP/DOPE lipoplexes were introduced in the water phase of the emulsion to form DNA LNC (Vonarbourg et al., 2009). Six temperature cycles were applied to reach phase inversion, between 20 and 60 °C, from an oil-in-water to a water-in-oil emulsion. Thereafter, the mixture underwent a fast cooling-dilution process with water at 0 °C, leading to the formation of LNCs in water.

2.1.3. Galactosylation of polymers

The synthesis of galactosylated F108 was performed by enzymatic galactosylation as already described (Letrou-Bonneval et al., 2008). The synthesis of DSPE-PEG-gal was performed by chemical galactosylation via a reductive amination which required lactose use. Under nitrogen atmosphere, lactose (245 mg, 716 μmol, 40 equiv.) was added at room temperature in 10 ml of anhydrous dichloromethane/methanol (1:1, v/v) and mixed with 50 mg of DSPE-PEG (18 μmol, 1 equiv.). The reaction mixture was stirred for 2 h at 50 °C. Then 28.2 mg of sodium cyanoborohydride (4.47 mmol, 25 equiv.) in methanol (200 μl) was added and the mixture was again heated at 50 °C for 4 h. The solution was concentrated under reduced pressure, diluted with phosphate buffer (pH 7, 0.12 M) and purified by dialysis (Cellu·Sep® H1 dialysis membrane 2,000 MCWO) against distilled water at 4 °C, followed by lyophilisation to afford DSPE-PEG-gal as a white solid (70 mg, up to 25% of galactose incorporation).

2.1.4. Preparation of coated and galactosylated DNA LNCs by the post-insertion method

Two kinds of polymers were used for post-insertion: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-$mPEG_{2000}$) (MW = 2805 g/mol) (Avanti Polar Lipids, Inc., Alabaster, USA) and Pluronic® F108 (poly(ethyleneoxide)$_{132}$-poly(propyleneoxide)$_{50}$-poly(ethyleneoxide)$_{132}$) (MW = 14,600 g/mol) kindly provided by BASF, galactosylated or not. These polymers were added to a fixed quantity of DNA LNCs by the post-insertion method (Hoarau et al., 2004; Uster et al., 1996) in order to obtain a final concentration of 2, 5 and 10 mM (DSPE-$mPEG_{2000}$ or DSPE-PEG_{2000}-gal) and 1, 2, 3 mM (F108 or F108-gal). Prior to post-insertion, the LNCs were purified thanks to the use of PD10 Sephadex columns (Amersham Biosciences Europe, Orsay, France) and then concentrated by ultrafiltration with Millipore Amicon® Ultra-15 centrifugal filter devices (Millipore, St Quentin-Yvelines, France). Preformed DNA LNCs were co-incubated for 4 h at 30 °C with DSPE-$mPEG_{2000}$ or F108 with or without galactose to form the so-called coated DNA LNCs or galactosylated DNA LNCs. The mixture was vortexed every 15 min and then quenched in an ice bath for 1 min. To provide controls, the same thermal treatment was applied to LNC suspensions without polymers.

2.2. Nanoparticle characterisation

2.2.1. Physico-chemical characteristics of coated DNA LNCs and galactosylated DNA LNCs

The average hydrodynamic diameter, the polydispersity index (PI) and the zeta potential of DNA LNCs were determined by dynamic light scattering (DLS), using a Malvern Zetasizer®

(Nano Serie DTS 1060, Malvern Instruments S.A., Worcestershire, UK). DNA LNCs were diluted 1:100 (v/v) in deionized water at 25 °C in order to assure convenient scatter intensity on the detector.

2.2.2. DNA stability study

The stability of nanocapsule suspensions during storage at 4 °C was assessed by measuring the size distribution. The stability was also tested after centrifugation at 15,000 × *g* at room temperature for 20 min in order to visualise separation among all the components. The stability of encapsulation and the integrity of DNA molecules after the process of nanocapsule formulation and post-insertion were evaluated by electrophoresis. A volume of LNC or lipoplex suspension equivalent to 0.2 μg of DNA before and after treatment with Triton® X100 (Sigma, Saint-Quentin Fallavier, France) was mixed with a gel-loading solution (Sigma, Saint-Quentin Fallavier, France) and deposited in each well of agarose gel 1% containing ethidium bromide (Sigma, Saint-Quentin Fallavier, France). Controls were constituted by 0.2 μg of free DNA in solution or associated to cationic lipids. Samples were left to migrate for 30 min at 100 V in Tris–EDTA buffer.

2.2.3. Galactose accessibility

Soybean lectin from Glycine max (Sigma, Saint-Quentin Fallavier, France) also called Soybean agglutinin (SBA) (1 mg/ml in PBS) was added to an equal volume of DNA LNCs, coated DNA LNCs (DNA LNCs + DSPE-mPEG$_{2000}$ 4 mM or F108 2 mM) or galactosylated DNA LNCs (DNA LNCs + DSPE-PEG$_{2000}$-galactose 4 mM or F108-galactose 2 mM). These concentrations of polymers were chosen to have the same number of galactose (648 galactose moieties) per nanocapsule whatever the tested polymer. Agglutination induced by SBA was monitored by measuring the turbidity of the solution at 450 nm using a spectrophotometer UV, Uvikon 922, (Kontron Instruments, Montigny Le Bretonneux, France).

To saturate galactose-binding sites on SBA, SBA was pre-incubated with D-galactose (100 μM) and an additional absorbance scan was performed after addition of the nanocapsules.

2.3. In vitro transfection studies

2.3.1. Cell line culture

The HeLa human cervical cancer cell line and H1299 lung cancer cell line were grown in high glucose DMEM (Invitrogen) (4.5 g/l). Cell culture media were supplemented with 10% FBS, 2 mM L-glutamine, 10 μg/ml streptomycin, 100 μ/ml penicillin at 37 °C in humid conditions with 5% CO_2. Cells were plated at a density of 35,000/cm^2 24 h prior to transfection in the same medium.

2.3.2. Primary culture of hepatocytes

Hepatocytes were isolated from the liver of fed, male rats or mice by the collagenase method (Berry and Friend, 1969) and modified as described elsewhere (Balavoine et al., 1993). Briefly, their livers were perfused with Hank's balanced salt solution (HBSS) and washed at a rate of 5 ml/min using the inferior *veina cava* before collagenase was added (0.025%). Dead cells were eliminated through a density gradient using Percoll®, and viable cells were plated at a density of 75,000/cm^2 on collagen-coated plates. Cells were given a time span of 2 h to attach themselves to William's medium E with Glutamax® (Invitrogen), 10% fetal bovine serum (FBS), 10 μg/ml streptomycin, 100 μ/ml penicillin, 100 nM dexamethasone and 100 nM insulin (Actrapid®, Novo Nordisk, Bagsvaerd, Denmark).

2.3.3. Transfection

Cells were transfected with DOTAP/DOPE-DNA lipoplexes formulated at DOTAP/DOPE-DNA ± charge ratio of 5 as a control. DNA LNCs containing 2 μg of plasmid-encoding luciferase were added to each well in the presence of F108, F108-gal, DSPE-mPEG$_{2000}$ or DSPE-PEG$_{2000}$-gal at their surface. Cells were cultured for 24 h in cell culture media supplemented with 10% FBS before gene expression was determined.

2.4. Luciferase assay

Luciferase activity was measured using the Promega luciferase assay system (Madison, WI, USA). Cells were rinsed twice with 500 μl of phosphate-buffered saline (PBS) and lysed with 200 μl of reporter lysis buffer (Roche Diagnostics, Mannheim, Germany) supplemented with a protease-inhibitor cocktail (Roche Diagnostics). Hepatocyte cells were then subjected to 4 freeze/thaw cycles. The cells were then centrifuged at 10,000 × *g* for 5 min at 4 °C before being assayed for luciferase activity. Each datum point represents the triplicate mean and is normalised to protein content. 20 μl of cellular homogenate supernatant was mixed with 100 μl luciferase assay buffer (Promega luciferase assay system, Madison, WI, USA). The luciferase activity was assayed by measuring light emission with a VICTOR^2 multilabel counter (PerkinElmer, Les Ulis, France) and the relative light units of each sample were counted for 10 s. Protein content was measured with a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Pierce, Brebières, France).

3. Results

3.1. Galactosylation of polymers

In regards with the method used for synthesized F108-gal (Letrou-Bonneval et al., 2008), the enzymatic galactosylation was not adapted to DSPE-aminoPEG$_{2000}$. Indeed, this kind of galactosylation uses the transglycosylation activity of the galactoside hydrolase from *Aspergillus oryzae*, and it utilizes the transfer of a glycosidically bound sugar to another hydroxyl group. Therefore, the synthesis of DSPE-PEG$_{2000}$-gal was performed by chemical galactosylation (Fig. 1) via a reductive amination which required lactose use: during the functionalisation, the first saccharose unit was opened providing galactose as the only targeting moiety. As the galactosylation was not complete, the average number of galactose units grafted onto steric stabilisers was determined by the ratio of the anomeric signal of galactose (1H, H1 gal, 4.30 ppm, d, J = 7.8 Hz) and the chemical shifts of non-modified terminal methylenes CH_2–O–H (δ = 3.75–3.80 ppm) of F108 or the non-modified terminal methylenes CH_2–NH_2 (δ = 4.01–3.90 ppm) of DSPE-aminoPEG$_{2000}$. This calculation indicated that around 25% of the terminal groups of the F108 (2 groups per molecule) and DSPE-aminoPEG$_{2000}$ (1 group per molecule) polymers were linked to galactose.

3.2. Formation of DNA LNCs, coated DNA LNCs and galactosylated DNA LNCs

As already described, lipid nanocapsules were slightly modified to encapsulate lipoplexes of DOTAP/DOPE (1/1 molar ratio) at a ± charge ratio of 5 in their lipid core (Vonarbourg et al., 2009). The thus-synthesized vector was called DNA LNCs. With the aim to synthesize coated DNA LNCs, PEG lipid derivatives DSPE-mPEG$_{2000}$ and F108 block copolymers were associated to preformed DNA LNCs by the post-insertion method, usually used to create stealth liposomes and recently applied to LNCs (Beduneau et al., 2007; Hoarau et al., 2004). The galactosylated DNA LNCs were synthesized following the same method but using galacosylated DSPE-PEG$_{2000}$ or galactosylated F108 (Fig. 2). The polymers were added at different concentrations (2, 5 and 10 mM for DSPE-PEG$_{2000}$ and 1, 2 and 3 mM of F108) to a constant concentration of preformed DNA LNCs.

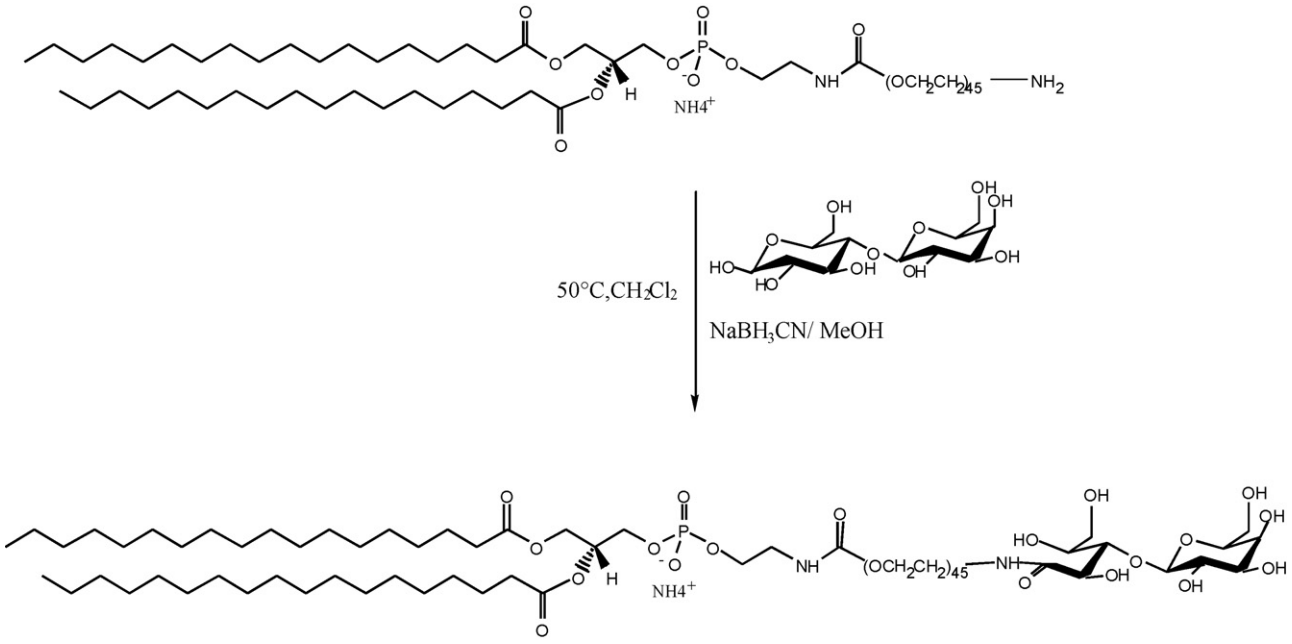

Fig. 1. Chemical synthesis of galactosylated DSPE-PEG$_{2000}$ via a reductive amination reaction. The synthesis of DSPE-PEG$_{2000}$-gal required lactose use providing the formation of lactosylated distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(amino polyethyleneglycol-2000) (DSPE-PEG-Lac), the glucose unit of the lactose was opened providing galactose as the only targeting moiety.

3.3. Physical characteristics of DNA LNCs, coated DNA LNCs and galactosylated DNA LNCs

DSPE-PEG$_{2000}$-gal and F108-gal densities at the surface of DNA LNCs were calculated as previously described (Lacoeuille et al., 2007; Vittaz et al., 1996) using mean diameter measurements ($A = 4\pi r^2$) and the molar concentration of the post-inserted polymers (Table 1). DSPE-mPEG$_{2000}$ concentrations from 2 to 10 mM (representing approximately 1297–6509 PEG chains per nanocapsule) and F108 concentrations from 1 to 3 mM (representing 1297–3890 PEG chains per nanocapsule) were used. As the chemo-enzymatic and chemical galactosylations were not total (25% of the terminal groups of F108 and DSPE-PEG$_{2000}$ were linked to galactose), we estimated that the number of galactose molecules per nanocapsule was estimated between 324 and 1627 for DSPE-PEG$_{2000}$-gal coated DNA LNCs, and between 324 and 972 for F108-gal coated DNA LNCs (Table 1).

The physico-chemical properties (Tables 1 and 2) and the DNA encapsulation ability (Fig. 3) of DNA LNCs were examined before and after the post-insertion of DSPE-mPEG$_{2000}$, DSPE-PEG$_{2000}$-gal, F108 or F108-gal. The coupling of DSPE-PEG$_{2000}$, DSPE-PEG$_{2000}$-gal, F108 or F108-gal slightly increased the size (Table 2), but this still resulted in vectors with a size inferior to 200 nm. Non-coated DNA LNCs exposed a size of 109 ± 6 nm and a zeta potential of +30 mV. The DSPE-mPEG$_{2000}$ coating led to the formation of nanoparticles with surface charge of +23, −12, −41 mV for 2, 5, 10 mM of DSPE-mPEG$_{2000}$, respectively. When coated with F108, the nanocapsules exposed a surface charge from +14 to +22 mV. The addition of galac-

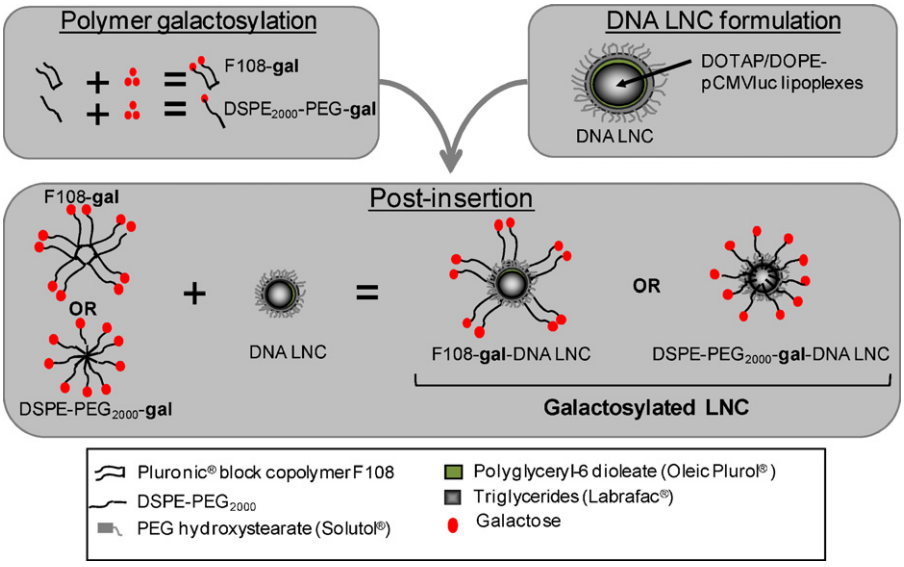

Fig. 2. Schematic representation of the formulation of galactosylated DNA LNCs. DSPE-PEG$_{2000}$ and F108 were galactosylated, to provide DSPE-PEG-gal and F108-gal. The hydrophobic moieties of the polymers (DSPE anchor of DSPE-PEG$_{2000}$-gal and poly(propyleneoxide) (PPO) part of F108-gal) were associated to the lipid nanocapsules, forming the so-called galactosylated DNA LNCs.

Table 1
Theoretical calculation of the number of coating molecules at the DNA LNC surface.

	DSPE-PEG$_{2000}$-gal (mM)			F108-gal (mM)		
	2	5	10	1	2	3
Surface density of PEG chains (PEG/nm^2)	0.04	0.10	0.20	0.04	0.08	0.12
Number of PEG chains per nanocapsule	1,297	3,254	6,509	1,297	2,592	3,890
Number of galactose per nanocapsule	324	813	1,627	324	648	972

Table 2
Influence of the incorporation of DSPE-mPEG$_{2000}$ and F108 at the surface of DNA LNCs on size, polydispersity and zeta potential. DNA LNCs, coated DNA LNCs and galactosylated DNA LNCs were analyzed for dynamic light scattering after the formulation and/or post-insertion process. Results show the mean ± SD of at least four independent formulations and three measurements per sample.

	Size ± SD (nm)	PdI	Zeta ± SD (mV)
DNA LNCs	109 ± 06	0.257	+31 ± 02
DSPE-mPEG$_{2000}$, 2 mM	131 ± 10	0.230	+23 ± 08
DSPE-mPEG$_{2000}$, 5 mM	139 ± 19	0.374	−12 ± 03
DSPE-mPEG$_{2000}$, 10 mM	142 ± 20	0.250	−41 ± 11
DSPE-PEG$_{2000}$-gal, 2 mM	132 ± 19	0.274	+23 ± 10
DSPE-PEG$_{2000}$-gal, 5 mM	136 ± 29	0.305	+22 ± 12
DSPE-PEG$_{2000}$-gal, 10 mM	172 ± 07	0.376	+26 ± 05
F108, 1 mM	117 ± 06	0.276	+14 ± 05
F108, 2 mM	139 ± 15	0.261	+17 ± 04
F108, 3 mM	138 ± 05	0.388	+22 ± 08
F108-gal, 1 mM	129 ± 03	0.289	+05 ± 03
F108-gal, 2 mM	138 ± 12	0.329	+13 ± 08
F108-gal, 3 mM	182 ± 21	0.426	+11 ± 09

tose at the extremity of DSPE-mPEG$_{2000}$ and F108 led to DNA LNCs with surface charges from +22 to +26 mV for DSPE-PEG$_{2000}$-gal and from +5 to +13 mV for F108-gal (Table 2).

Agarose gel electrophoresis experiments showed that DNA molecules did not migrate after the galactosylated nanocapsule formulation process (Fig. 3). By contrast, incubation of nanocapsules with Triton® X100 led to the release of DNA molecules that migrated into the gel. These results clearly indicated that the addition of galactosylated polymers at the surface of DNA LNCs did not disturb the encapsulation of DNA (lanes 16–27) and that DNA molecules were still well protected inside the nanocapsules, as already observed for the post-insertion of non-galactosylated polymers (lanes 4–15).

3.4. Galactose accessibility

To assess the accessibility of the galactose residues at the DNA LNCs surface, we monitored the binding of DNA LNCs to a galactose specific lectin, the Soybean agglutinin (SBA) by measuring the absorbance of the suspension at 450 nm: when a specific agglutination occurred between nanoparticles and SBA, these aggregates induced an increase in absorbance. As shown in Fig. 4, the turbidity was not significantly different between nanoparticles incubated with SBA alone and nanoparticles incubated with SBA + D-galactose for DNA LNCs and coated DNA LNCs with the two polymers. This tends to prove that there was no specific targeting of SBA with these formulations. A slight but significant increase in absorbance was observed for DNA LNCs coated with DSPE-PEG$_{2000}$-gal compared to DNA LNCs coated with non-galactosylated DSPE-PEG$_{2000}$ (0.175 vs. 0.135). The presence of F108-gal at the surface of DNA LNCs induced a strong increase in absorbance (0.637 vs. 0.202 with non-galactosylated F108 coated DNA LNCs) implying a specific agglutination with SBA. This association was not observed when SBA was pre-incubated with D-galactose, confirming that the interaction between complexes and the lectin is specific, and occurred through the galactose-binding sites of SBA. Moreover, a non-specific interaction exists between F108 and SBA (0.385 with SBA galactose-binding sites blocked with an excess of galactose). Nevertheless, these results demonstrated that galactose residues were well-displayed at the surface of DNA LNCs coated with F108-gal.

3.5. Transfection efficiency

The transfection ability of DNA LNCs and polymer-coated DNA LNCs, encapsulating pCMVluciferase as a reporter gene, was investigated in HeLa human cervical and H1299 lung cancer cell lines used as model cells by measuring luciferase activity (Fig. 5a). Since these cells are not expressing ASPG-R, the galactosylated nanocapsules were not tested here. Lipoplexes were used as positive controls. First, DNA LNC transfection efficiency was found to be at the same scale as this control (around 200 ng of luciferase/protein mg). Significantly higher transfection efficiency was observed with F108-coated DNA LNCs, whatever the concentration, both in HeLa and H1299 cells, with a maximum of luciferase expression for 2 mM F108 (223 ng luciferase/protein mg for H1299 and 353 ng luciferase/protein mg for HeLa). As observed here, the transfection efficiency of F108-coated DNA LNCs in HeLa was slightly superior to those of H1299. By contrast, DSPE-mPEG$_{2000}$ DNA LNCs did not lead to significant luciferase expression, with a maximum of only 4 ng luciferase/protein mg expressed in H1299 cells, and 32 ng luciferase/protein mg in HeLa cells.

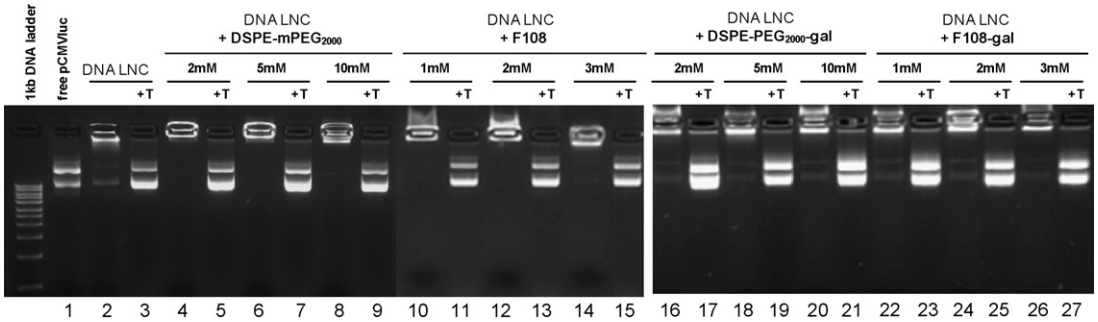

Fig. 3. Agarose gel electrophoresis. The influence of coating on the encapsulation efficiency was tested for all the types of DNA LNC suspensions: coated DNA LNCs (lanes 4–15), galactosylated DNA LNCs (lanes 16–27). DNA molecules cannot migrate once encapsulated in nanocapsules (lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26), contrary to free DNA (pCMVluciferase) (lane1). The incubation of nanocapsules with Triton® X100 (+T) led to the release of DNA molecules that migrated into the gel (lane 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27).

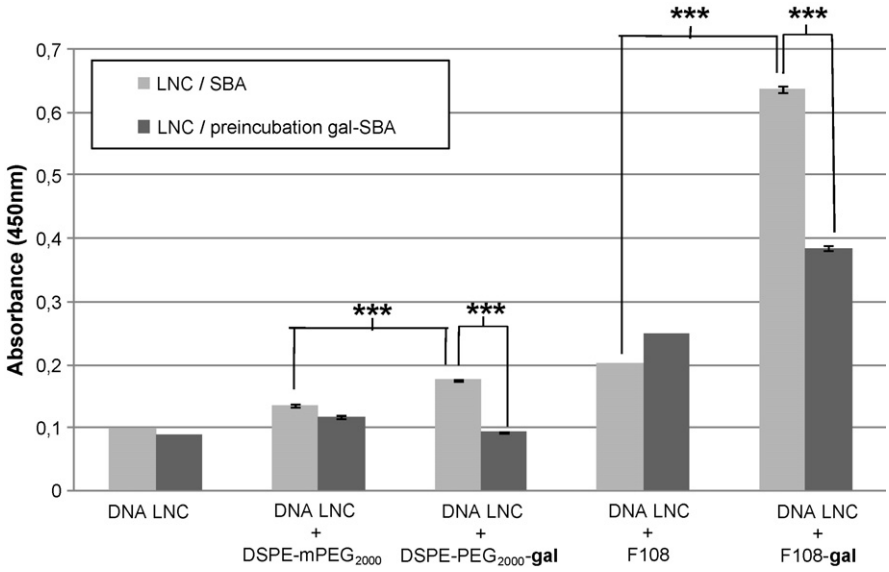

Fig. 4. Observation of lectin-induced agglutination of DNA LNCs, coated DNA LNCs and galactosylated DNA LNCs. The absorbance at 450 nm of a solution containing the nanoparticles incubated with Soybean agglutinin (SBA) was measured (grey). As a control, SBA was pre-incubated with an excess of free D-galactose, to saturate all the galactose-binding sites present on SBA, and nanocapsules were added in a second time (dark grey) before measuring the absorbance. $^{**}P < 0.01$ (Dunnett test).

We next investigated the influence of active *in vitro* targeting using galactosylated DNA LNCs on primary hepatocyte cells (Fig. 5b), which have numerous ASPG-Rs expressed at the surface. To prove receptor-mediated transfection efficiency, the difference of behaviour between non-galactosylated and galactosylated DNA LNCs was compared. DNA LNCs without coating exposed a naturally good tendency to transfect primary hepatocytes (2240 ng luciferase/protein mg), almost equivalent to that of DOTAP/DOPE

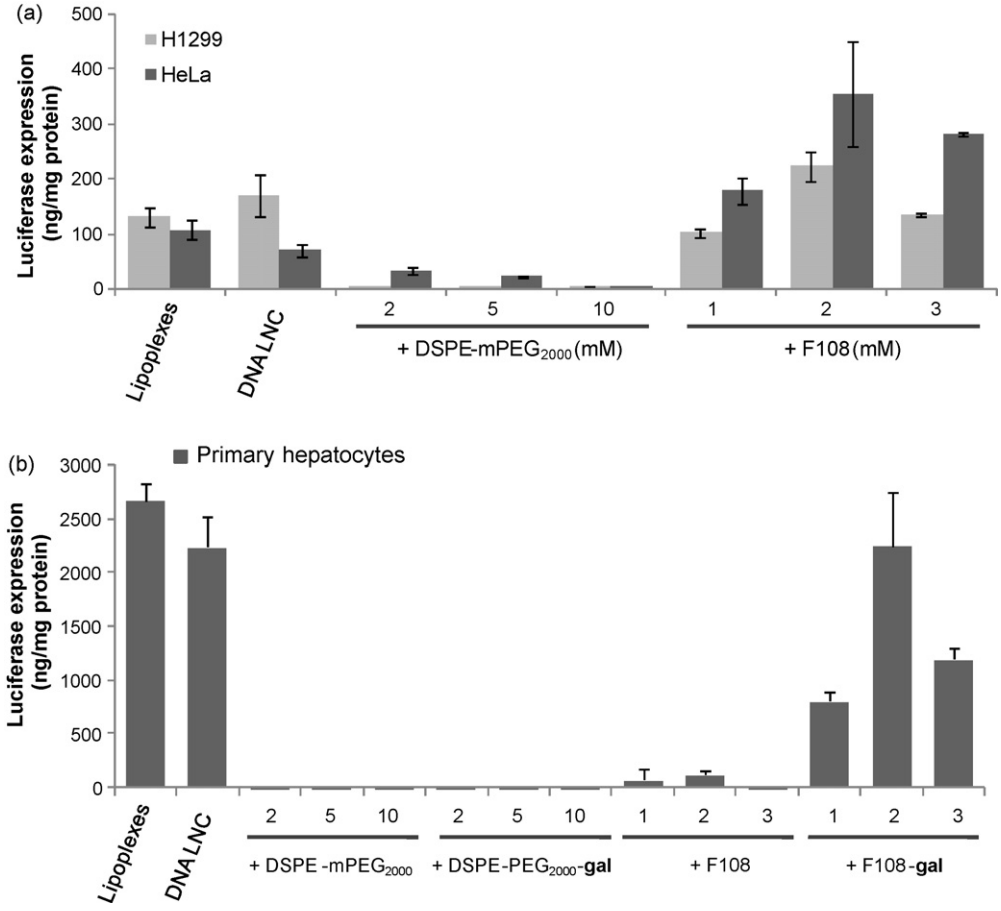

Fig. 5. *In vitro* transfection activity of DNA LNCs (a) in tumour cell lines. HeLa and H1299 cells were incubated with DNA LNCs and DSPE-mPEG$_{2000}$- or F108-coated DNA LNCs, and DOTAP/DOPE lipoplexes were used as a positive control. The results are given in ng of luciferase per protein mg. Values are shown as the mean ± SD ($n = 3$) (b) in primary hepatocytes. Cells were incubated with DNA LNCs, coated DNA LNCs, or galactosylated DNA LNCs. DOTAP/DOPE lipoplexes were tested as a positive control. The results are given in ng of luciferase per protein mg. Values are shown as the mean ± SD ($n = 3$).

lipoplexes (2672 ng luciferase/protein mg). This efficiency was also found with the galactosylated F108 coating at the concentration of 2 mM (2247 ng luciferase/protein mg), with an 18-fold increase in luciferase expression compared to non-galactosylated 2 mM F108 DNA LNCs. By contrast, the attachment of galactose at the extremity of DSPE-PEG_{2000} did not show any increase in transfection efficiency compared to non-galactosylated DSPE-PEG_{2000}. Indeed, with or without galactose, the luciferase expression did not exceed 10 ng of luciferase per mg of proteins for this kind of coating.

4. Discussion

The specific delivery of a therapeutic gene to the liver is of great importance since hepatocytes (i.e., parenchymal liver cells) are key targets for the secretion of gene products in the systemic circulation system. However, systemic targeting remains a real challenge and a compromise has to be found between sufficient circulation time, non-toxicity and transfection efficiency on targeted cells. The significant increase in plasmatic circulation time in mice due to the shield created by PEG around DNA LNCs via DSPE-$mPEG_{2000}$ or F108 polymers (submitted results) could be sufficient to reach the desired organs, especially the endothelial fenestrae in the liver which constitute an open communication lane for circulating gene transfer vectors to the Disse space and hence provide subsequent access and uptake in hepatocytes (Braet and Wisse, 2002). The chance of gene expression in these cells could therefore be facilitated (Nishikawa et al., 1998; Sato et al., 2007).

Zeta potential measurements revealed that DNA LNCs were able to mask partially the positive charge due to the presence of the lipoplexes (Table 2). The negative values obtained with the use of DSPE-$mPEG_{2000}$ could be attributed to the presence of negative PEG dipoles that can form a mushroom/brush intermediate PEG conformation as already described by Vonarbourg et al. (2006a). When galactose molecules were added at the extremity of post-inserted polymers, the dipole effect was cancelled in case of DSPE-PEG_{2000} proving that galactose can interact with the PEG chains. In a different way, the shielding of lipoplexe charges was accentuated in F108 case, which is in favour of the presence of galactose at the extremity of the polymer chains (Table 2).

To check if the galactose displayed at the DNA LNC surface was accessible and recognizable by a galactose receptor, galactosylated DNA LNCs were confronted to soybean lectin, a tetrameric glycoprotein containing four galactose-binding sites (Tsuda et al., 1975). This study confirmed that galactose moieties were accessible at the surface of galactosylated DNA LNCs coated with F108. This effect was much less pronounced with galactosylated DNA LNCs coated by DSPE-PEG_{2000}. This difference in galactose exposition between the two polymers could be explained by a difference in PEG conformation and PEG length. Indeed, the PEG chain density as calculated at the surface of DNA LNCs could lead to a spatial conformation called mushroom/brush intermediate or mushroom like (Vonarbourg et al., 2006a). Therefore, if the DSPE-PEG_{2000} (45 PEG units) chains are in such a folded conformation, their distal end, bearing the galactose moiety, will be hidden inside the short PEG_{660} chains already present at the surface of DNA LNC (15 PEG units). By contrast, the length of each PEG chains on F108 is more important (135 PEG unit per chain), and even if these PEG chains are submitted to a mushroom like conformation, the galactose will be more distant from the highly PEGylated environment present at the surface of DNA LNCs, and will remain more accessible to its receptor.

Then, the ability of *in vitro* transfection of non-galactosylated long-circulating carriers was tested on HeLa and H1299 cell line models (Fig. 5a). It was interesting to note that comparable *in vitro* transfection was observed for DOTAP/DOPE lipoplexes (well known as efficient *in vitro* transfection reagents) and non-coated DNA LNCs. As DNA LNCs exposed positive surface charge, the interaction with the cell membrane could be facilitated (Mislick and Baldeschwieler, 1996; Mounkes et al., 1998). Moreover, this positive surface charge could help the endosomal escape thanks to a physical disruption of the negatively charged endosomal membrane occurring on direct interaction with the positively charged cationic vector, as suggested for both PAMAM dendrimers and poly(L-lysine) (Zhang and Smith, 2000). Regarding the sterically stabilised DNA LNCs with DSPE-$mPEG_{2000}$, transfection efficiency failed in comparison to non-coated DNA LNCs (by 42-fold in H1299 and by 2-fold in HeLa cells). In HeLa cells, this decrease was in correlation with the increase in DSPE-$mPEG_{2000}$ coating concentration (Fig. 3). This result is in good agreement with the well-known drawback of PEG, and it is probably due to the prevention of the association of DSPE-$mPEG_{2000}$ coated DNA LNCs with cell membranes and/or to PEG-inhibition of endosomal escape linked to their lower surface charge (compared to non-coated DNA LNCs) (Shi et al., 2002; Song et al., 2002). Indeed, the strong association between DNA LNCs and DSPE-PEG_{2000} due to lipid anchoring in the LNC core (Hoarau et al., 2004) and the negative surface charge (for DSPE-$mPEG_{2000}$ 5 and 10 mM) could result in an impossible dissociation of endosomes and a subsequent degradation of the vector in lysosomes. By contrast, F108 block copolymer coating led to a significant increase in luciferase expression, with an optimal concentration of 2 mM representing 1297 F108 molecules per nanocapsule. This transfection efficiency could be explained by the presence of poly(propyleneoxide) (PPO) segments which can provide improved interaction of these molecules with biological membranes (Alakhov and Kabanov, 1998). Indeed, block copolymers have recently been seen to have considerable promise for the delivery of pDNA, thanks to their proven *in vivo* transfection efficiency (Desigaux et al., 2005; Kabanov et al., 2002; Pitard et al., 2002; Richard et al., 2005a,b).

With the goal of evaluating if steric hindrance induced by PEG could be overcome by galactose attachment, we tested galactosylated DNA LNCs on primary hepatocytes. As observed for cell line transfection studies (Fig. 5a), DNA LNCs were as efficient in transfecting hepatocytes as DOTAP/DOPE lipoplexes (Fig. 5b). The luciferase expression was significantly more important in these primary cultured cells compared to the cancer cell lines (i.e. luciferase expression 32-fold more important in primary hepatocytes compared to HeLa cell line for non-coated DNA LNCs). The transfection efficiency of DNA LNCs seems therefore to be cell-dependant, as observed for other gene-delivery systems, i.e. chitosan DNA nanoparticles (Mao et al., 2001). However, with the goal of systemic delivery, the coating of DNA LNCs is required *in vivo*, as the $t_{1/2}$ of elimination of DNA LNCs was too low in mice (data not shown), mainly due to their positive charge (31 ± 2 mV). As expected in regards with the degree of accessibility of galactose when attached to F108 (Fig. 4), the F108-gal coating was found to strongly improve gene delivery in primary hepatocytes (2247 ng luciferase/protein mg), compared to F108 without galactose which exposed low transfection efficiency (121 ng luciferase/protein mg). Nevertheless, the grafting of galactose to DSPE-PEG_{2000} molecules did not improve transfection efficiency, probably due to a non-sufficient accessibility of galactose when attached to this polymer (Fig. 4). Indeed, as described in the literature, it seems important to place ligands several nanometers away from the surface of the particle in order to provide effective binding to cell surface receptors (Blessing et al., 2001) and the DSPE-PEG_{2000} chains are probably in an inadequate conformation to present their distal end to the ASPG-R. The presence of free DSPE-PEG_{2000}-gal could also provide, by a competitive process for the ASPG-R, a decrease in transfection efficiency.

We have demonstrated here that DNA LNCs can be used to achieve targeted-gene expression based on a cell-specific, receptor-mediated, endocytosis process, when coated with F108-gal with an optimal concentration at 2 mM. This kind of nanoparticles could

therefore allow gene targeting to hepatocytes by systemic injection thanks to their circulating properties, and provide a promising systemic gene-delivery system.

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