

Galactosylated multimodular lipoplexes for specific gene transfer into primary hepatocytes

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Abstract

Background Numerous synthetic cationic vectors have been synthesized and are successfully used for *in vitro* gene transfer but an excess of positive charges can lead to cytotoxicity and does not enable specific transfection.

Methods We decided to develop alternative molecular systems consisting of neutral, colloiddally stable bioassemblies equipped with ligands for specific cell targeting. Consequently, we directed our efforts toward the development of a multimodular non-viral gene delivery system consisting of a condensed core of DNA with cationic liposomes of bis(guanidinium)-tren-cholesterol and an external corona of poly(ethylene oxide) stretches harbored by the steric stabilizers used to stabilize lipoplexes colloiddally. A ligand capable of cell targeting by receptor-mediated endocytosis was covalently linked at the poly(ethylene oxide) extremity of steric stabilizers. Steric stabilizers were functionalized by a one-step enzymatic galactosylation to develop new supramolecular assemblies of lipoplexes able to target asialoglycoprotein receptors located on primary hepatocytes.

Results Cryo-TEM and fluorescence experiments showed that DNA was condensed within lamellar complexes whose size ranged between 100 to 300 nm in diameter. Bis(guanidinium)-tren-cholesterol-DNA lipoplexes, colloiddally stabilized by galactosylated steric stabilizers at a galactosylated steric stabilizer/DNA ratio of 300, led to specific transfection of primary hepatocytes whereas ungalactosylated steric stabilizer did not transfect.

Conclusions Our findings confirm the receptor-mediated endocytosis pathway of galactosylated multimodular lipoplexes. Thus, we conclude that the fabrication of a multimodular assembly harboring a ligand without non-specific interaction with cell membranes is possible and a highly promising system to transfect other primary or cultured cells specifically through a receptor-dependent mechanism. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords asialoglycoprotein receptor; block copolymer; endocytosis; galactose; gene transfer; hepatocytes

Introduction

Since 1987, numerous cationic vectors have been synthesized and are currently used for delivery of nucleic acids into cultured cells [1,2]. The principle of non-viral gene delivery relies on the interaction of nucleic acids with cationic residues present on the vector through electrostatic forces [1,3]. The resulting cationic vector/DNA complexes display a three-stage model of colloiddal stability depending on the theoretical charge ratio, corresponding

to the moles of positive charges per mole of negative phosphates [4,5]. In zone A, for charge ratios below 1, negatively charged stable complexes are formed. In zone B, complexes precipitate as the charge ratio is close to neutral (ranging from 1 to 3, in the present study) due to the absence of electrostatic repulsion forces. In zone C (for charge ratios above 4, in the present study), complexes are colloidally stable through electrostatic repulsion due to positively charged complexes. The morphology depends on the theoretical charge ratio but multilamellar structures are usually observed in cationic lipid/DNA lipoplexes irrespective of the charge ratio [2]. The most active complexes are those from zone C because their electrostatic interactions with negative molecules present at the cell surface lead to non-specific internalization through endocytosis, phagocytosis or macropinocytosis processes [3,6]. Whereas many cells types are transfected with cationic lipid/DNA lipoplexes using this principle, the excess of cationic lipid used to generate positively charged lipoplexes can lead to high cytotoxicity, compromising cell viability [7,8] and preventing specific transfection. In this context, numerous strategies have been described to limit the cytotoxicity of positively charged complexes such as decreasing their charge ratio [9–11]. However, the transfection activity of cationic vectors/DNA complexes was less efficient because they did not spontaneously internalize into cells by electrostatic interactions. Thus, various studies [9–12] have tried to substitute non-specific binding of positively charged particles to cells with a more specific interaction by a receptor-mediated process. The asialoglycoprotein receptor (ASPGR), present at the surface of hepatocytes, has been one of the most used glycosylated receptors for the targeting of cationic vector-based gene delivery systems [13]. ASPGR naturally binds and internalizes terminal galactose-bearing asialoglycoproteins [14]. Thus, galactose molecules were grafted onto different cationic vectors, including cationic lipids, polyethyleneimines (PEI) [15–18], polylysine (PLL) [19–22] and peptides [23], to target ASPGR specifically. Some studies have reported the synthesis of lipid molecules with headgroups containing both cationic polyamines and galactose residues [9,10,12]. In order to form colloidally stable DNA complexes with this type of molecule, positively charged lipoplexes were used to transfect a human hepatoma cell line (HepG2). In this context, the presence of galactose in the cationic lipid led to a modest increase in transfection compared to that obtained with cationic lipids without galactose. This was due to the still positive zeta potential of lipoplexes even with galactosylated cationic lipid, which led to non-specific transfection. Remy *et al.* [11] described the formation of particles resulting from the association of DNA with two independent molecules, i.e. cationic lipopolyamine and galactosylated neutral lipid. Transfection of the HepG2 cell line showed that these particles led to a 1000-fold increase in transgene expression compared to lipopolyamine/DNA lipoplexes without galactosylated neutral lipid. However, addition of asialofetuin, the natural ligand of ASPGR, did not lead to

a decrease in transgene expression, questioning the mechanism of transfection of the particles. Other approaches to transfect the HepG2 cell line specifically were explored with cationic polymers including PLL [19–22] and PEI [15–18]. Galactosylated PLL (PLL-gal) was shown to increase transfection efficiency compared to parent PLL but the presence of residual positive amines in the PLL backbone prevented non-specific transfection activity from being completely abolished [19–22]. Moreover, addition of chloroquine was necessary to observe transfection, with PLL either galactosylated or not [21]. Therefore, the same strategy was employed with PEI, which does not require chloroquine to transfect cultured cells. The galactose-bearing PEI (PEI-gal) was prepared by reductive amination between PEI and the galactosyl residue with an optimal percentage of galactosylation reported to be 5% of the total amine functions in PEI [15,16,18]. Nonetheless, around neutrality, particles became much larger and unstable, so physicochemical properties like particle size and electric charge were investigated [15]. In spite of efforts at downsizing, the galactosylated complexes still presented a slightly positively charged surface. Therefore, a modest increase in gene transfection in HepG2 cells was observed with galactosylated particles compared to ungalactosylated ones [15–17,20]. Indeed, again the presence of residual positive charges at the surface of galactosylated particles tends to show that complexes are internalized not only by receptor-mediated endocytosis, but also non-specific endocytosis by electrostatic interactions. These observations prompted us to design and evaluate new supramolecular assemblies capable of only specific nucleic acid delivery. More precisely, our goal here was to explore the efficiency of multimodular assemblies containing a condensed core of DNA surrounded by a corona of poly(ethylene oxide) stretches linked to hydrophobic moieties functionalized with ligand at the distal ends for cell recognition and internalization by a receptor-mediated process. Thus, we first designed a simple and fast synthetic procedure to produce poly(ethylene oxide) hydrophobic derivatives containing galactose residues to target the ASPGR on primary hepatocytes. As complexes have to be close to neutrality to avoid non-specific interaction with cell membranes, we decided to form small complexes resulting from the association of bis(guanidinium)-tren-cholesterol (BGTC)/dioleylphosphatidylethanolamine (DOPE) liposomes and DNA at a theoretical charge ratio close to neutrality in the presence of functionalized poly(ethylene oxide) hydrophobic derivatives, which serves here both as a steric colloidal stabilizer and to target receptors. In the present study, we demonstrate that it is possible to fabricate galactosylated multimodular lipoplexes by self-assembly of BGTC/DOPE liposomes, DNA and steric colloidal stabilizer with chemical tunability. We also report the results of cryo-transmission electron microscopy experiments showing that the morphology of the multimodular supramolecular assemblies consisted of ordered lamellar domains. Finally, we report data from *in vitro* gene expression studies demonstrating that

galactosylated multimodular lipoplexes are efficient for transfection in primary hepatocytes in a specific manner and that non-equipped particles were totally inefficient.

Materials and methods

Plasmids, steric stabilizers and cationic liposomes

pCMV-Luc [24] is a plasmid encoding the luciferase reporter gene under the control of the human cytomegalovirus immediate-early gene promoter. Plasmid was purified from recombinant *E. coli* by means of Endo-free plasmid purification columns (Qiagen, Chatsworth, CA, USA). Steric stabilizers (Scheme 1), F68 (80% poly(ethylene oxide); molecular weight (MW) 8400) and F108 (80% poly(ethylene oxide), MW 14600), were generously provided by BASF. Stock solutions of steric stabilizers were prepared at given weight-to-weight (w/w) concentration in sterilized water and were kept at 4 °C. BGTC/DOPE liposomes were obtained as previously described [5].

Enzymatic galactosylation of steric stabilizers

The synthesis of F68-gal and F108-gal was performed by first dissolving F68 and F108 (1 mM) in acetate buffer (100 mM, pH 4.5) and in *N,N*-dimethylformamide (10%). Then *p*-nitrophenyl β -D-galactopyranoside (pNP- β -gal) (Sigma-Aldrich; 2 mmol) was added at room temperature under vigorous stirring. Finally, the β -galactosidase from *Aspergillus oryzae* (Sigma-Aldrich; 8.9 units) was added to the remaining solution, and the mixture was stirred for 15 h at 30 °C. The reaction was stopped by heating at 100 °C for 5 min, and the mixture was immediately dialyzed for 4 days under distilled water using a Pierce SnakeSkin® membrane (molecular weight cutoff (MWCO) 3500 Da). Then, the products were centrifuged, filtered with Amicon Ultra-15 centrifugal filter units (Millipore; MWCO 30.000 Da) and freeze-dried. F68-gal: ^1H NMR (D_2O): δ 4.30 (H1 gal, d, $J = 7.8$ Hz), 3.78–3.73 ($\text{CH}_2\text{-CH}_2\text{-O-H}$, m), 3.67–3.40 (bs, $(\text{CH}_2\text{-CH}_2\text{-O})_n$, $(\text{-O-CH}_2\text{-CH-(CH}_3\text{)-O})_n$), 1.06–1.03 (m, $(\text{-O-CH}_2\text{-CH-(CH}_3\text{)-O})_n$). F108-gal: ^1H NMR (D_2O): δ 4.30 (H1 gal, d, $J = 7.8$ Hz), 3.80–3.75 ($\text{CH}_2\text{-CH}_2\text{-O-H}$, m), 3.67–3.29 (bs, $(\text{CH}_2\text{-CH}_2\text{-O})_n$,

$(\text{-O-CH}_2\text{-CH-(CH}_3\text{)-O})_n$), 1.09–1.06 (m, $(\text{-O-CH}_2\text{-CH-(CH}_3\text{)-O})_n$).

Preparation of lipoplexes

Formulations of DNA with BGTC were prepared by mixing equal volumes of various concentrations of BGTC/DOPE liposomes with plasmid DNA at the desired concentration. Formulations of DNA complexes with F108, F108-gal or F68, F68-gal were prepared as described above by mixing solutions of cationic liposomes containing various concentrations of steric stabilizers with plasmid DNA solution.

Dynamic light scattering, fluorescence and cryo-TEM studies

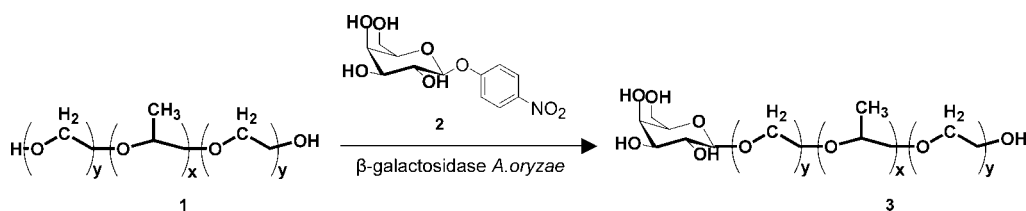
Dynamic light scattering measurements were made on a Zetasizer 3000 HSA (Malvern) at 20 °C as previously described [4,5]. Samples were prepared by complexing DNA with BGTC/DOPE liposomes at a charge ratio of 2 and with F108-gal or F108 at various w/w ratios. Formation of complexes was confirmed by electrophoresis on 1.0% agarose gel with Tris-acetate running buffer at 100 V for 20 min. Fluorescence and cryo-TEM experiments were performed as previously described [4,5,25].

Size determination of complexes in transfection media

BGTC/DOPE-DNA/F108-gal lipoplexes prepared at a charge ratio of 2 and at an F108-gal/DNA ratio (w/w) of 300 and BGTC/DOPE-DNA lipoplexes at a charge ratio of 4 were formed in 150 mM NaCl by mixing 50 μl of F108-gal or BGTC/DOPE solutions with 50 μl (200 $\mu\text{g/ml}$) plasmid DNA. Samples were analyzed after 20 min of complexation at room temperature. Just prior to dynamic light scattering analysis, 1000 μl of either 150 mM NaCl or Dulbecco's modified Eagle's medium (DMEM) (plus additives used for culturing primary hepatocytes) was added to lipoplexes. Size determination was obtained every hour for lipoplexes diluted with NaCl or DMEM.

Primary culture of hepatocytes

Hepatocytes were isolated from the liver of fed male rats or mice by the collagenase method [26], modified as



Scheme 1. Enzymatic synthesis of galactosylated steric stabilizers 3 by means of the transgalactosylation activity of a galactoside hydrolase

described elsewhere [27,28]. Briefly, livers were perfused with Hank's balanced salt solution, and washed at a rate of 5 ml/min using the inferior vena cava before collagenase (0.025%) was added. Dead cells were eliminated through a density gradient using percoll, and viable cells were plated at a density of 75 000/cm² on collagen-coated plates. Cells were given a time span of 2 h to attach in William's medium E with Glutamax (Invitrogen), 10% fetal bovine serum (FBS), 10 µg/ml streptomycin, 100 u/ml penicillin, 100 nM dexamethasone and 100 nM insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark).

Cell line culture

Cos7 green monkey kidney fibroblast cells 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 u/ml penicillin at 37 °C under humidified 5% CO₂. Cells were plated at a density of 35 000/cm² 24 h prior to transfection.

Culture cell transfection

Cells were transfected with BGTC/DOPE-DNA lipoplexes formulated at BGTC/DOPE-DNA charge ratios of 4 or 2 in the presence of F108-gal, F108 or F68-gal, F68 at various steric stabilizer/DNA ratios. BGTC/DOPE-DNA/steric stabilizer lipoplexes containing 2 µg of luciferase plasmid were added to each well. Lipoplexes were incubated at room temperature for 15–20 min before transfection. After transfection (4 h), the serum-free medium (300 µl) was then replaced with 1 ml of growth medium containing 10% FBS for Cos7 and without FBS for primary hepatocytes. Cells were cultured for an additional 20 h before gene expression was determined.

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). Then, strictly hepatocyte cells were subjected to four freeze/thaw cycles. After 5 min of centrifugation at 10 000 rpm and 4 °C, luciferase activities were measured from an aliquot of supernatant with a VICTOR² multilabel counter (Perkin Elmer, Les Ulis, France). Luciferase activity was assayed by measuring light emission after addition of 100 µl of luciferase substrate to 20 µl of the supernatant. Protein content was measured with a bicinchoninic acid (BCA) protein assay kit.

Formation and transfection with YOYO-1-labeled plasmid

YOYO-1 was from Molecular Probes (Eugene, USA). The luciferase plasmid was added to a solution of YOYO-1 (0.1 mM in dimethyl sulfoxide (DMSO)) at the desired mixing ratio (dye/base pairs). A dye/base pair ratio 1 : 150 is equivalent to the addition of one molecule of dye for every 150 base pairs of plasmid DNA. The mixture was incubated at room temperature for 10 min before self-assembly of BGTC/DOPE-DNA lipoplexes formulated at a BGTC/DOPE-DNA charge ratio of 2 in the presence of F108-gal at an F108-gal/DNA ratio of 300 or in the presence of F108 at an F108/DNA ratio of 300; or with a BGTC/DOPE-DNA charge ratio of 4. Primary hepatocytes were incubated with lipoplexes for 4 h. After transfection, the serum-free medium (300 µl) was replaced with fresh medium. Then, 20 h post-transfection, cells were washed twice with 500 µl of PBS and fixed with 4% paraformaldehyde for 30 min at 4 °C. Finally, cells were rinsed again with PBS and mounted with fluorescent mounting medium (DAKO, Carpenterina, CA, USA) for observation. Cells were observed under FITC filter to determine the YOYO internalization.

Results

Galactosylation of steric stabilizers

The grafting of galactosyl residues onto two steric stabilizers, F68 and F108, was performed by a chemo-enzymatic glycosylation (Scheme 1) through the transglycosylation activity of the galactoside hydrolase from *Aspergillus oryzae* using pNP-β-gal as donor and steric stabilizers as acceptors. As this method was based on using the transfer activity of galactoside hydrolase, we used a large excess of donor to favor transglycosylation products [29]. Galactosylated F108 (F108-gal) and galactosylated F68 (F68-gal) were characterized by different methods. First, an aqueous size-exclusion chromatography method was performed on ungalactosylated and galactosylated steric stabilizers to evaluate the polydispersity of the steric stabilizers. Both chromatograms were similar, indicating that the size distribution of steric stabilizers was not modified by the enzymatic reaction (data not shown). F108-gal and F68-gal were also analyzed by H¹ NMR at 400 MHz using D₂O as the lock solvent. The anomeric proton of the galactose unit (H1 gal, 4.30ppm, d) presents a *J* coupling constant near 7–8 Hz, which is characteristic of a β grafting. Therefore, enzymatic synthesis led strictly to β-F108-gal and β-F68-gal and did not modify the polydispersity of steric stabilizers. As the galactosylation was not complete, the average number of galactose units grafted onto steric stabilizers 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 (4H, CH₂-CH₂-O-H, 3.75–3.80 ppm,

m) of steric stabilizers. This calculation indicated that around 25% of the terminal OH groups of the F108 and F68 steric stabilizers were linked to β -galactose.

Galactosylated multimodular lipoplexes

Next, galactosylated multimodular lipoplexes were assembled by condensing DNA molecules with BGTC/DOPE liposomes at a charge ratio close to neutrality in the presence of F108-gal or F108. A thorough understanding of

the physicochemical properties of lipoplexes as a function of the steric stabilizer/DNA ratio is crucial for the self-assembly of the gene delivery system. Therefore, we examined the influence of this factor on the colloidal stability of the BGTC/DOPE-DNA/F108-gal lipoplexes and the BGTC/DOPE-DNA/F108 lipoplexes. To this end, we measured the mean particle diameter by dynamic light scattering. Figure 1A illustrates the mean diameter of BGTC/DOPE-DNA lipoplexes at a charge ratio of 2 as a function of F108-gal/DNA ratio and F108/DNA ratio. As previously shown [30], in the absence of steric stabilizer and at this low ratio, we obtained colloiddally

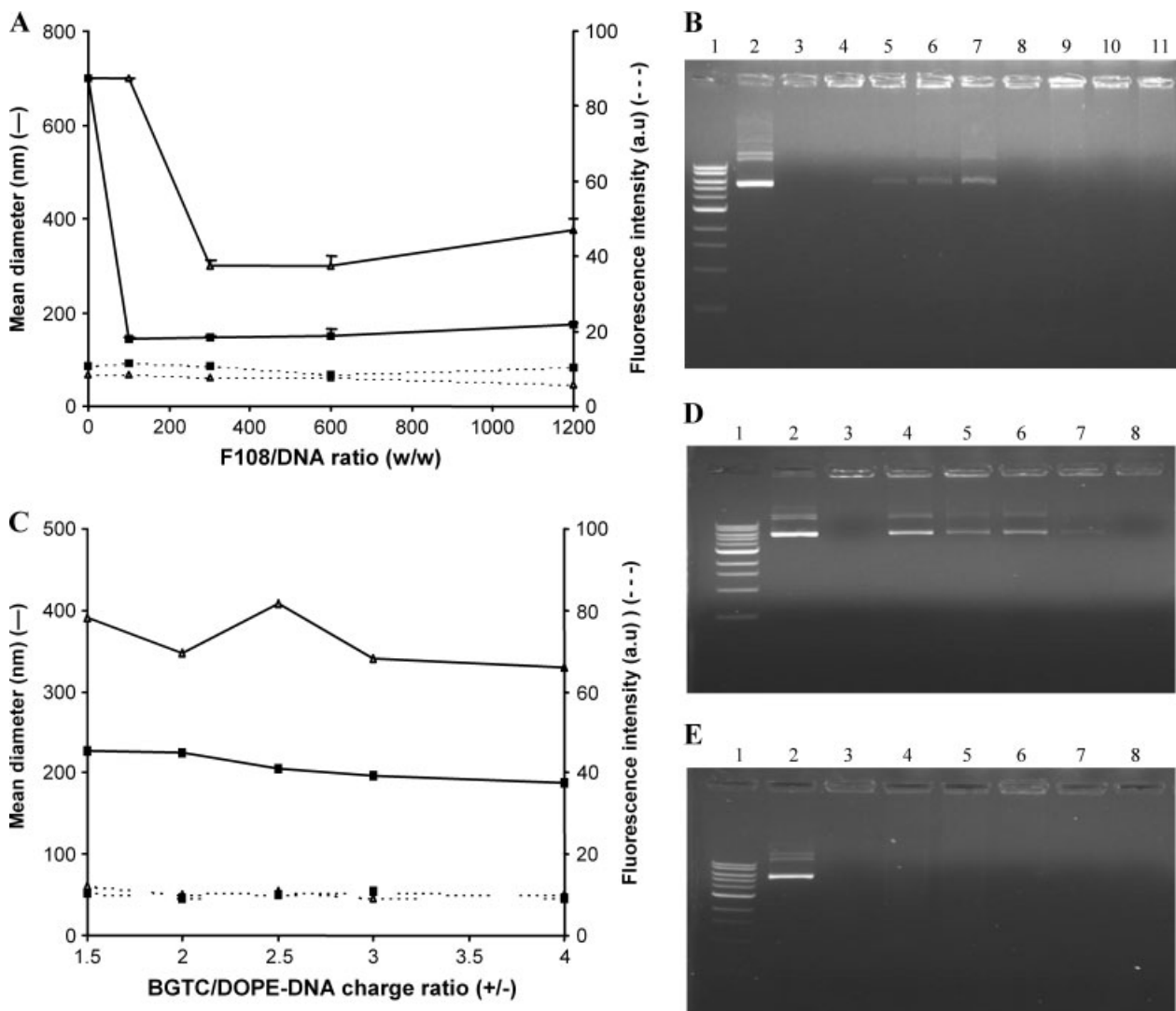


Figure 1. DNA complexation and colloidal stabilization of BGTC/DOPE-DNA lipoplexes in the presence of F108-gal or F108. To assess the colloidal stability of lipoplexes and nucleic acid complexation, dynamic light scattering (solid lines) and BET fluorescence measurements (dashed lines) were performed after 20 min of complexation, respectively. (A) Mean diameter and BET fluorescence of lipoplexes as a function of F108-gal/DNA (w/w) ratio (Δ) and F108/DNA (w/w) ratio (\blacksquare). (B) Agarose gel electrophoresis of BGTC/DOPE-DNA/F108-gal lipoplexes as a function of F108-gal/DNA (w/w) ratio and BGTC/DOPE-DNA/F108 lipoplexes as a function of F108/DNA (w/w) ratio. Lanes 1, 2 and 3 correspond to a 1 kb DNA ladder, naked plasmid, and aggregated BGTC/DOPE-DNA lipoplexes, respectively. Lanes 4–7 and 8–11 correspond to BGTC/DOPE-DNA lipoplexes formed in the presence of F108-gal and F108, respectively. (C) Colloidal stability and DNA complexation of BGTC/DOPE-DNA lipoplexes with F108-gal (Δ) and F108 (\blacksquare) as a function of the BGTC/DOPE-/DNA charge ratio. (D) Agarose gel electrophoresis of BGTC/DOPE-DNA/F108-gal lipoplexes at an F108-gal/DNA ratio of 300 as a function of various BGTC/DOPE-DNA charge ratios and (E) BGTC/DOPE-DNA/F108 lipoplexes at an F108/DNA ratio of 300 as a function of various BGTC/DOPE-DNA ratios. Lanes 1, 2 and 3 correspond to a 1 kb DNA ladder, naked plasmid, and BGTC/DOPE-DNA lipoplexes at a charge ratio of 2, respectively. Lanes 4–8 correspond to BGTC/DOPE-DNA lipoplexes at a BGTC/DOPE-DNA charge ratio of 1.5, 2, 2.5, 3 and 4, respectively

unstable lipoplexes, as evidenced by visible precipitates which cannot be measured by dynamic light scattering. Under this condition an arbitrary value of 700 nm was attributed for those non-colloidally stable lipoplexes. By contrast, addition of F108 allowed the formation of stable DNA complexes with a mean diameter of 150 nm at an F108/DNA ratio starting from 100. F108-gal led also to the formation of colloidally stable BGTC/DOPE-DNA lipoplexes with a mean diameter of 300 nm starting from an F108-gal/DNA ratio of 300. Fluorescence experiments, performed by exposing BGTC/DOPE-DNA/F108-gal lipoplexes and BGTC/DOPE-DNA/F108 lipoplexes to ethidium bromide, showed that the fluorescence level was very low. This indicates that all DNA molecules were totally incorporated into BGTC/DOPE-DNA particles even stabilized by F108-gal or F108. To confirm these results, DNA entrapment into lipoplexes of various F108-gal/DNA and F108/DNA ratios was assayed by agarose gel electrophoresis experiments. The results presented in Figure 1B demonstrate that DNA molecules associated with BGTC/DOPE liposomes in the presence of F108 did not migrate into the gel, although, in the presence of F108-gal, lipoplexes were partially destabilized during gel electrophoresis experiment as evidenced by some DNA

molecules which were able to migrate into the gel for the highest F108-gal/DNA ratio. Next, we studied the influence of the BGTC/DOPE-DNA charge ratio on the mean diameter of complexes and the DNA complexation in the presence of F108-gal or F108. Figure 1C shows that DNA complexed by BGTC/DOPE liposomes in the presence of F108-gal and F108 led to the formation of particles of a mean diameter of about 350 and 220 nm, respectively, irrespective of the BGTC/DOPE-DNA charge ratio. BET intercalating experiments indicated that the fluorescence signal was at its minimal value for both galactosylated and ungalactosylated particles, although the agarose gel electrophoresis analysis indicated (Figures 1D and 1E) that BGTC/DOPE-DNA/F108-gal lipoplexes for BGTC/DOPE-DNA ratios ranging from 1.5 to 3 (Figure 1D) were partially destabilized during the experiment.

Cryo-TEM of galactosylated multimodular lipoplexes

The supramolecular assemblies of BGTC/DOPE-DNA lipoplexes in the presence of F108 and F108-gal were observed by cryo-transmission electron microscopy (cryo-TEM) (Figure 2). In the presence of F108 at an

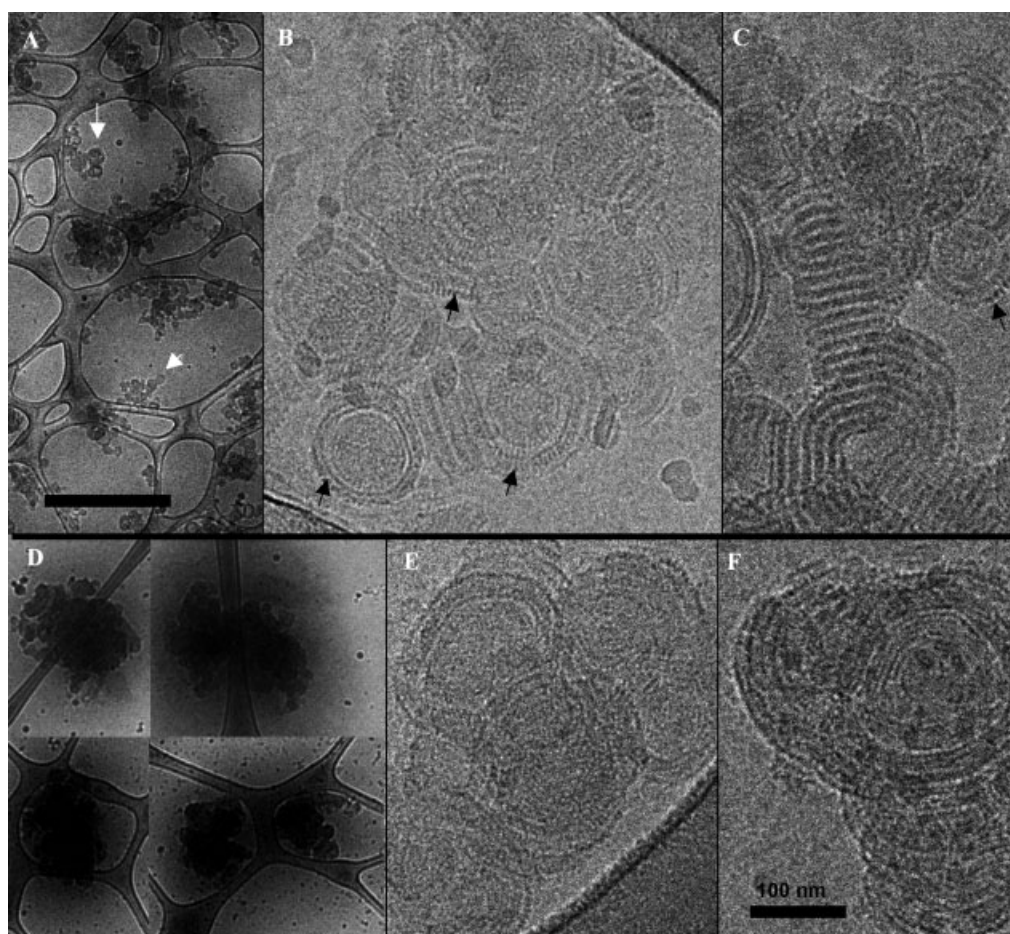


Figure 2. Cryo-TEM visualization of multimodular lipoplexes. (A) Field of view of BGTC/DOPE-DNA/F108 lipoplexes. (B, C) Images at high magnification of lipoplexes showing complex assemblies of DNA sandwiched between lipid membranes (black arrows). (D) Gallery of images of BGTC/DOPE-DNA/F108-gal lipoplexes revealing dense and large assemblies. (E, F) Images at high magnification of a small part of the DNA condensate. Scale bars 1 μ m (A, D) and 100 nm (B, C, E, F)

F108/DNA ratio of 300, lipoplexes ranged in size from 50 to 300 nm and were mainly composed of grapes of liposome-sized structures (white arrows). Indeed, spherical structures made of liposomes surrounded by DNA strands were involved in the formation of larger aggregates (Figure 2B). The 2.5 nm regular striation visible at the liposome surface corresponded to the assembly of DNA strands (black arrows). Lipoplexes containing the typical repeat structure of DNA/lipid bilayers involving a large restructuring of liposome were also visible (Figure 2C). The lipoplexes formed in the presence of F108-gal at an F108-gal/DNA ratio of 300 led to larger lipoplexes, which were encountered on the grid in small quantities (a few lipoplexes per square grid). These lipoplexes (shown in a gallery of images) appeared larger and denser than those obtained with F108, their sizes ranging from 200 to 500 nm (Figure 2D). Due to their larger size, only the periphery of the lipoplexes can be correctly imaged at higher magnification (Figures 2E and 2F). The structures exhibited a regular repeat corresponding to DNA strands sandwiched between lipid bilayers and revealed a significant lipid membrane rearrangement. Although their overall size was different, the lipoplexes exhibited comparable organization in the presence of both F108 and F108-gal; thus it is very likely that these steric stabilizers have no noticeable influence on intimate BGTC/DOPE–DNA interactions.

Colloidal behavior of lipoplexes in transfection medium

Dynamic light scattering analysis of lipoplexes provides a means of checking for the colloidal stability of particles destined for *in vitro* transfection. However, measurement of particle size alone does not take into account possible transfection medium-induced effects on the size of the complexes. In fact, it is possible that charged species in the medium could alter the size of preformed lipoplexes before effective transfection takes place [31]. To address this issue, preformed lipoplexes representing both BGTC/DOPE-DNA/F108-gal lipoplexes prepared at a charge ratio of 2 and at an F108-gal/DNA ratio of 300, and BGTC/DOPE-DNA lipoplexes at a charge ratio of 4, were evaluated either in the transfection medium of primary hepatocytes or in 150 mM NaCl solution. Results from dynamic light scattering analysis performed every hour on the lipoplexes are shown in Figure 3. It can be seen that, within 1 h of adding transfection medium, the size of lipoplexes at a BGTC/DOPE-DNA charge ratio of 4 began to increase until aggregates were formed. By contrast, BGTC/DOPE-DNA/F108-gal lipoplexes were able to maintain their small size in saline solution as well as in transfection medium.

Biological activity of galactosylated multimodular lipoplexes

We next investigated the ability of BGTC/DOPE-DNA/F108-gal lipoplexes or BGTC/DOPE-DNA/F68-gal

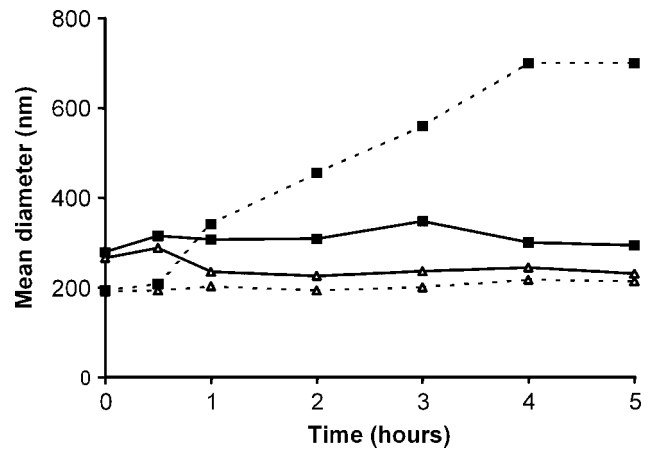


Figure 3. Colloidal stability of lipoplexes in transfection medium. Dynamic light scattering analysis of positively charged BGTC/DOPE-DNA lipoplexes (dashed lines) at a BGTC/DOPE-DNA charge ratio of 4 was performed either in primary hepatocyte transfection medium (■) or in 150 mM NaCl (△). Similar experiments with BGTC/DOPE-DNA/F108-gal lipoplexes (solid lines) at a BGTC/DOPE-DNA charge ratio of 2 and at an F108-gal/DNA ratio of 300 (w/w) were conducted; i.e. primary hepatocytes transfection medium (■) and NaCl (△)

lipoplexes to transfect primary hepatocyte cells. Primary hepatocyte cells were transfected with BGTC/DOPE-DNA lipoplexes formulated at a BGTC/DOPE-DNA charge ratio of 2 in the presence of F108-gal and F108 or F68-gal and F68 at various steric stabilizer/DNA ratios. Figure 4A shows that DNA complexes formulated with F108-gal led to a dramatic increase in luciferase expression as the F108-gal/DNA ratio increased. The same trend was also observed with F68-gal but led to a lower luciferase expression. By contrast, BGTC/DOPE-DNA lipoplexes formed in the absence or presence of ungalactosylated F108 or F68 did not lead to significant primary hepatocyte transfection. Therefore, these results strongly suggest that particles equipped with galactose lead to specific hepatocyte transfection and that the length of the steric stabilizer plays a role both in the formation of supramolecular assemblies and in the accessibility of the ligand for the receptor-mediated endocytosis pathway. Further experiments were performed with particles characterized by an F108/DNA ratio of 300. Next, experiments were undertaken to determine the optimal BGTC/DOPE-DNA charge ratio that allowed the highest luciferase activity with BGTC/DOPE-DNA/F108-gal lipoplexes. Figure 4B illustrates that BGTC/DOPE-DNA/F108-gal lipoplexes formulated at a BGTC/DOPE-DNA charge ratio of 2 gave the highest transfection efficiency. We next evaluated the influence of the amount of DNA entrapped within sterically stabilized lipoplexes on the luciferase expression of transfected hepatocytes. Figure 4C indicates that luciferase activity was maximal for a DNA amount of 2 µg/well. In these two experiments (Figures 4B and 4C) ungalactosylated lipoplexes did not transfect primary hepatocytes. To demonstrate the cell-specific transfection with galactosylated multimodular lipoplexes, we carried out experiments on primary hepatocytes in the absence

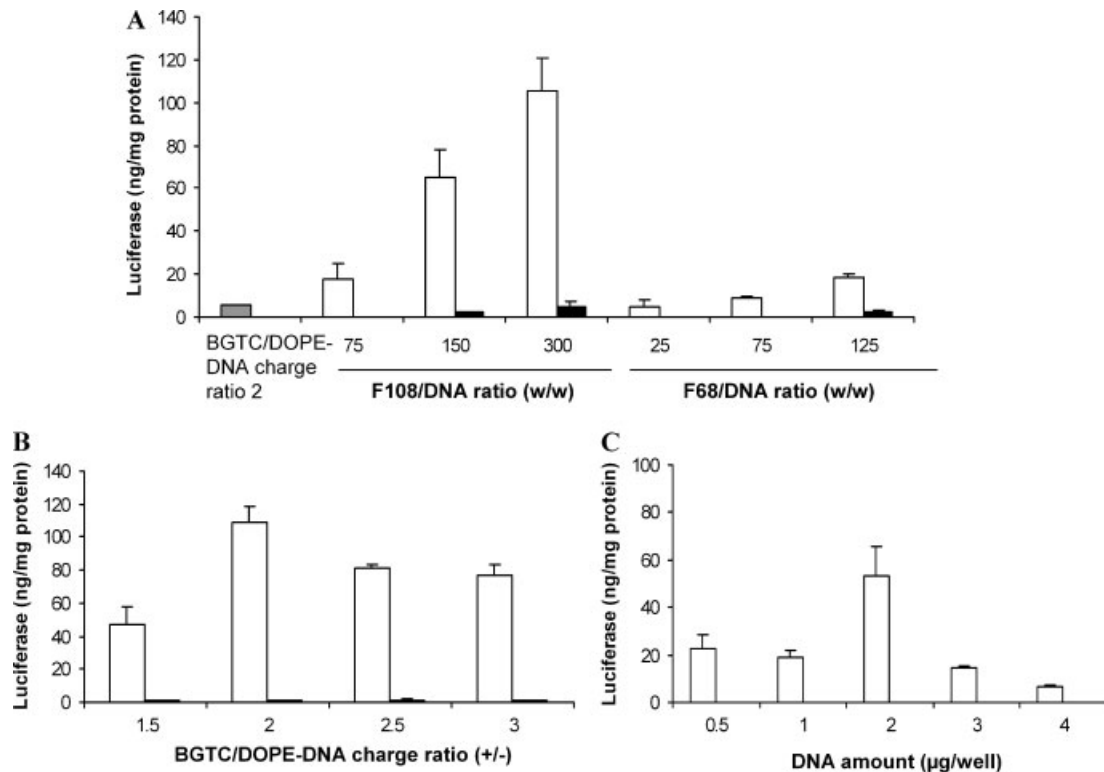


Figure 4. Transfection efficiency in primary hepatocytes of BGTC/DOPE-DNA/steric stabilizer lipoplexes as a function of (A) the chemical structure of the steric stabilizer, (B) the BGTC/DOPE-DNA charge ratio, and (C) the DNA amount. *In vitro*, luciferase activity in transfected primary hepatocytes was performed with galactosylated steric stabilizer (white bars) or ungalactosylated steric stabilizer (black bars). (A) Two different steric stabilizers with different chemical structures were galactosylated and used for the formation of BGTC/DOPE-DNA lipoplexes at a charge ratio of 2 and containing 40 µg DNA/ml. (B) BGTC/DOPE-DNA lipoplexes were prepared at various BGTC/DOPE-DNA charge ratios with F108-gal at an F108-gal/DNA (w/w) ratio of 300 or with F108 at an F108/DNA (w/w) ratio of 300 and 40 µg DNA/ml. (C) BGTC/DOPE-DNA lipoplexes were characterized by an F108-gal/DNA (w/w) ratio of 300, a BGTC/DOPE-DNA charge ratio of 2 and a DNA concentration ranging from 10 to 80 µg/ml

and presence of various concentrations of free galactose, which can bind to the ASGPR (Figure 5A). This experiment was also performed with BGTC/DOPE-DNA lipoplexes formed at a charge ratio of 4 as control because these positively charged lipoplexes should not be internalized through ASGPR but rather through electrostatic interactions. In the absence of free galactose, BGTC/DOPE-DNA/F108-gal lipoplexes at an F108-gal/DNA ratio of 300 formulated with BGTC/DOPE-DNA at a charge ratio of 2 gave a similar activity to BGTC/DOPE-DNA lipoplexes at a charge ratio of 4 (Figure 5A). We also observed that increasing the concentration of free galactose led to a progressive decrease in luciferase expression. By contrast, as expected the presence of free galactose did not affect the luciferase activity of highly positively charged lipoplexes (Figure 5A). Consequently, these results show that competition takes place between galactosylated multimodular lipoplexes and free galactose to interact with ASGPR, suggesting that BGTC/DOPE-DNA/F108-gal lipoplexes are internalized in primary hepatocytes by a receptor-mediated process. To confirm the specific effects of BGTC/DOPE-DNA/F108-gal lipoplexes on receptor-mediated transfection, we carried out control experiments on Cos7 cells, which do not express ASGPR. The transfection efficiency of lipoplexes colloiddally stabilized with F108-gal or F108 was similar and lower than

that observed with positively charged BGTC/DOPE-DNA lipoplexes (Figure 5B). To confirm the receptor-mediated endocytosis pathway for transfection of BGTC/DOPE-DNA/F108-gal lipoplexes, primary hepatocyte cells were transfected after pre-incubation with nocodazole, which induces depolymerization of microtubules. Nocodazole inhibits transport of endosomal vesicles from early to late endosomes. This chemical substance prevents lysosomal degradation so nocodazole improves one step of the endocytosis process. Nocodazole pre-treatment increased the transfection efficiency of BGTC/DOPE-DNA/F108-gal lipoplexes (Figure 5C). By contrast, in the presence or absence of nocodazole, BGTC/DOPE-DNA lipoplexes at a charge ratio of 4 gave similar levels of luciferase expression (data not shown). Therefore, BGTC/DOPE-DNA lipoplexes at a charge ratio of 4 are mainly internalized by non-specific electrostatic interactions with the cellular membrane, although BGTC/DOPE-DNA/F108-gal lipoplexes transfected through an endocytosis process.

Cellular uptake of galactosylated multimodular lipoplexes

We finally examined the cellular uptake of BGTC/DOPE-DNA/F108-gal lipoplexes into primary hepatocytes. The

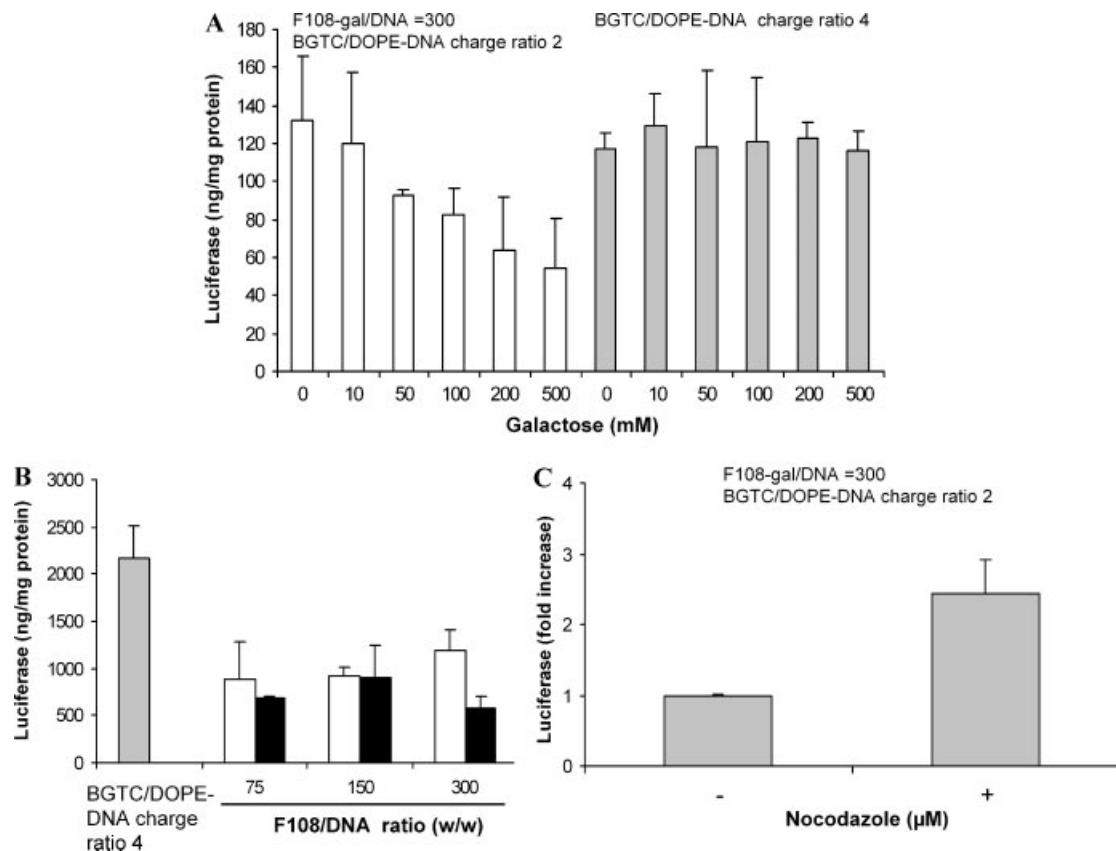


Figure 5. BGTC/DOPE-DNA/F108-gal lipoplexes enabled specific *in vitro* transfection. (A) Luciferase expression in primary hepatocyte cells of BGTC/DOPE-DNA lipoplexes either colloiddally stabilized with F108-gal (F108-gal/DNA (w/w) ratio of 300) or with positively charged particles (BGTC/DOPE-DNA charge ratio of 4) as a function of the concentration of free galactose added to the cell culture medium 2 h before the transfection. (B) Luciferase expression in Cos7 cells of positively charged BGTC/DOPE-DNA lipoplexes and BGTC/DOPE-DNA lipoplexes at a BGTC/DOPE-DNA charge ratio of 2 formed in the presence of F108-gal (white bars) or F108 (black bars). (C) Luciferase expression in primary hepatocyte cells of BGTC/DOPE-DNA/F108-gal lipoplexes characterized by a BGTC/DOPE-DNA charge ratio of 2, an F108-gal/DNA (w/w) ratio of 300 and 40 µg DNA/ml as a function of the pre-incubation during 45 min with 33 µM nocodazole

BGTC/DOPE-DNA/F108-gal lipoplexes, BGTC/DOPE-DNA/F108 lipoplexes and positively charged BGTC/DOPE-DNA lipoplexes were labeled with the fluorescent DNA-intercalating dye YOYO-1. Fluorescent complexes were incubated for 4 h with cells. The cells were then observed by microscopy. As shown in Figure 6A, BGTC/DOPE-DNA/F108-gal lipoplexes were detected inside almost all of the cells. In contrast, for cells incubated with BGTC/DOPE-DNA/F108 lipoplexes, no fluorescence signal was observed inside cells (Figure 6B). As a positive control, positively charged BGTC/DOPE-DNA lipoplexes were also shown in cells (Figure 6C).

Discussion

Over the past decade, numerous non-viral cationic vectors have been synthesized. They share a high efficiency for gene transfer *in vitro* [1,2]. Their use relies on the presence of a large excess of positive charges at the surface of cationic vectors/DNA complexes which is necessary to display a very high transfection activity [1,3]. Nonetheless, positive charges of complexes can lead

to toxicity and to non-specific interactions with the cell surface [7,8]. However, efforts to further mask residual positive charges present at the surface of complexes and to improve specific gene delivery via covalently linked residues aimed at receptor binding have so far failed. Thus, in the present study, we have investigated the relationships between the physicochemical properties and transfection efficiency in primary hepatocytes of DNA complexes formed by novel multimodular supramolecular assemblies. These are characterized by a condensed core of DNA surrounded by a corona of neutral steric stabilizers harboring galactose residues. We used a cationic lipid, BGTC, constituted of guanidinium residues as polar headgroup and cholesterol for the hydrophobic moiety to create the condensed core of particles close to neutrality (at a BGTC/DOPE-DNA charge ratio of 2). However, at this low charge ratio, we obtained BGTC/DOPE-DNA lipoplexes that were not colloiddally stable due to the absence of electrostatic repulsion forces. To colloiddally stabilize neutral lipoplexes, we used steric stabilizers (i.e. F68 and F108) which are poly(ethylene oxide) hydrophobic derivatives. Galactose residue was covalently linked at the distal end of the F108 and F68 for specific

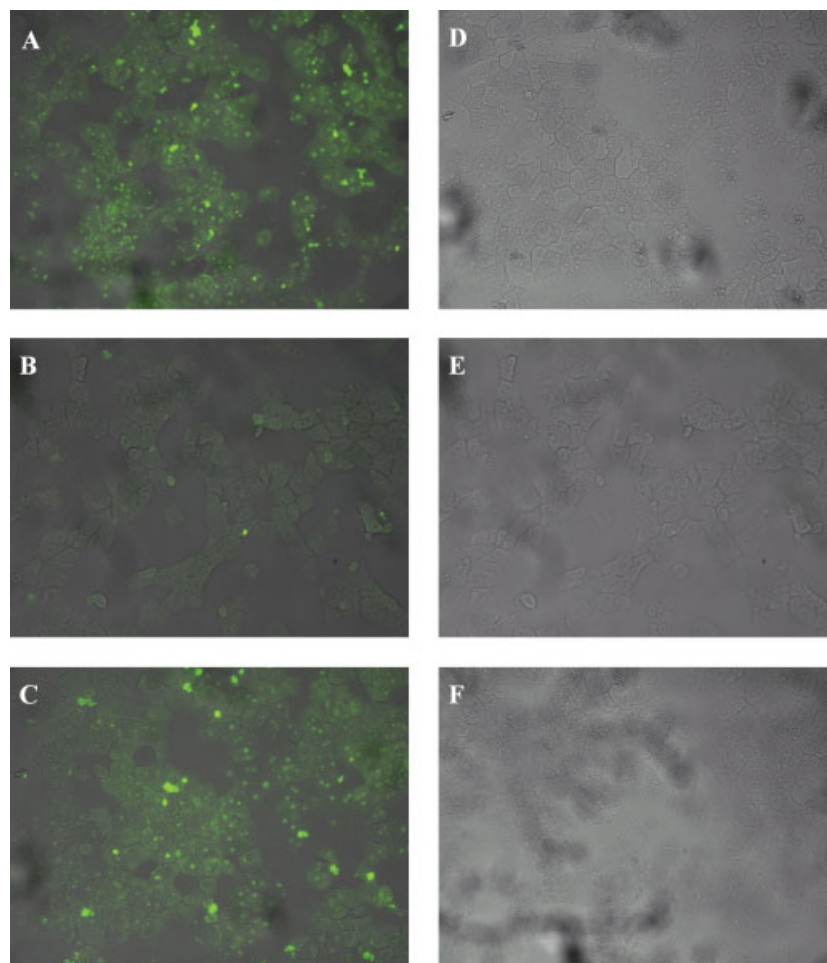


Figure 6. Primary hepatocyte cellular uptake of BGTC/DOPE-DNA/F108-gal lipoplexes (A, D), BGTC/DOPE-DNA/F108 (B, E) and positively charged BGTC/DOPE-DNA lipoplexes (C, F). DNA molecules were labeled with YOYO (1 molecule every 150 base pairs) before self-assembly with BGTC/DOPE liposomes at a charge ratio of 2 (A, B, D, E) or at a charge ratio of 4 (C, F). Primary hepatocytes were observed under FITC filter to see the YOYO fluorescence (A–C) and by phase contrast (D–E)

cell recognition. Most of the systems based on galactose-bearing cationic polymers (i.e. PEI-gal, PLL-gal) have been synthesized by chemical synthesis, which required at least three steps and led to a low percentage of grafted galactose molecules [15,16,18,19,21]. Indeed, direct functionalization of PEI by reductive amination between PEI and galactosyl residues led to an optimal percentage of galactosylation reported to be still only 5% of the total amine functions in PEI [15,16,18]. Therefore, to improve the limiting step of grafting of galactose units onto polymers, we have developed an enzymatic strategy for galactosylation using β -galactosidase from *Aspergillus oryzae*, which leads to the glycosylation of 25% of the accessible OH groups of F108 and F68. F108-gal led to colloidal stabilization of the condensed core of BGTC/DOPE-DNA lipoplexes. However, while F108 formed particles about 150 nm in diameter, F108-gal led to particles of about 300 nm in diameter. The same observation was noticed with PEI-gal, which led to bigger particles than unmodified PEI [16]. Therefore, it seemed that the presence of galactose modified the natural physicochemical properties of the vectors. Nevertheless, in our case, F108-gal did not perturb the

interaction between DNA and BGTC/DOPE liposomes leading to the formation of lamellar organization as observed with F108. The most active BGTC/DOPE-DNA/F108-gal lipoplexes for transfection of primary hepatocytes were characterized by a neutral charge ratio and an F108-gal/DNA ratio of 300 (w/w), and a certain lability as evidenced by the release of DNA molecules during gel agarose electrophoresis of complexes. This DNA release would be due to the destabilization of galactosylated lipoplexes by non-covalent interactions including Van der Waals, hydrogen or dipolar binding between galactosyl residues present in the agarose gel and galactosyl residues linked to the terminal OH group of F108, enhanced by the electric field. This lability characteristic of the multimodular lipoplexes may contribute to the intracellular disassembly of vector/DNA complexes and escape from vesicles before the DNA is degraded in late endosomes or lysosomes. The biological activity of the self-assembly galactosylated multimodular lipoplexes described in this study was investigated in rat primary hepatocytes rather than in the human hepatoma HepG2 cell line commonly used for specific targeting of the ASPGR. Results showed that only galactosylated

particles led to transfection. The absence of transfection observed with particles containing the condensed core of DNA surrounded by ungalactosylated F108 contrasts radically to that obtained with the control usually described in the literature, which led to residual non-specific transfection due to positive charges remaining on particles [9,10,12,15–17,21,22]. Remy *et al.* [11] also reported a multimodular gene delivery system combining two lipids; one positively charged and the other one containing galactose residues that led to transfection of a highly dividing human hepatoma cell line. In the present study, we have shown that the galactosylated multimodular lipoplexes specifically transfect primary hepatocytes expressing ASPGR as shown by galactose inhibition of the transfection. Total inhibition was not achieved even at high galactose concentration (0.5 M), since multimodular lipoplexes presented several galactose units at their surface, which induced avidity effects. The galactose density at the surface of BGTC/DOPE-DNA/F108-gal lipoplexes was estimated by assuming that the amount of steric stabilizer present per surface unit of lipoplex was 338 nmol/cm², according to the formula (n/N)/S, where n is the amount of steric stabilizer in solution (40.6 nmol), N is the number of lipoplexes in solution (1.25 × 10⁸), and S is the surface of one lipoplex (9.6 × 10⁻¹⁰ cm²). Therefore, these lipoplexes contained 135 nmol galactose/cm². This value was in good agreement with a previously described system characterized by a similar galactose density which enabled the binding of ASPGR of primary hepatocytes with a high affinity [32]. On the other hand, Remy *et al.* [11] did not succeed in decreasing the transfection efficiency of HepG2 in the presence of the natural ligand of ASPGR, i.e. asialofetuin. However, the absence of physicochemical characterization of their particles could suggest that novel supramolecular assemblies could be obtained involving a mechanism independent of the recognition by ASPGR. Therefore, the present work suggests that this versatile multimodular system appears to be a promising artificial method for specific gene delivery to ASPGR on primary hepatocyte cells and could be adapted to transfect other primary cells through specific recognition of receptors and to deliver other classes of nucleic acids including siRNA.

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References

- Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc Natl Acad Sci U S A* 1996; **93**: 12349–12354.
- Pitard B. Supramolecular assemblies of DNA delivery systems. *Somat Cell Mol Genet* 2002; **27**: 5–15.
- Labat-Moleur F, Steffan AM, Brisson C, *et al.* An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Ther* 1996; **3**: 1010–1017.
- Pitard B, Aguerre O, Airiau M, *et al.* Virus-sized self-assembling lamellar complexes between plasmid DNA and cationic micelles promote gene transfer. *Proc Natl Acad Sci U S A* 1997; **94**: 14412–14417.
- Pitard B, Oudrhiri N, Vigneron JP, *et al.* Structural characteristics of supramolecular assemblies formed by guanidinium-cholesterol reagents for gene transfection. *Proc Natl Acad Sci U S A* 1999; **96**: 2621–2626.
- Grosse S, Aron Y, Thévenot G, François D, Monsigny M, Fajac I. Potocytosis and cellular exit of complexes as cellular pathways for gene delivery by polycations. *J Gene Med* 2005; **7**: 1275–1286.
- Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc Natl Acad Sci U S A* 1989; **86**: 6982–6986.
- Felgner JH, Kumar R, Sridhar CN, *et al.* Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J Biol Chem* 1994; **269**: 2550–2561.
- Frisch B, Carrière M, Largeau C, *et al.* A new triantennary galactose-targeted PEGylated gene carrier, characterization of its complex with DNA, and transfection of hepatoma cells. *Bioconjug Chem* 2004; **15**: 754–764.
- Gaucheron J, Santaella C, Vierling P. In vitro gene transfer with a novel galactosylated spermine bolaamphiphile. *Bioconjugate Chem* 2001; **12**: 569–575.
- Remy JS, Kichler A, Mordvinov V, Schuber F, Behr JP. Targeted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles presenting galactose ligands: a stage toward artificial viruses. *Proc Natl Acad Sci U S A* 1995; **92**: 1744–178.
- Kawakami S, Yamashita F, Nishikawa M, Takakura Y, Hashida M. Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes. *Biochem Biophys Res Commun* 1998; **252**: 78–83.
- Schwartz AL. The hepatic asialoglycoprotein receptor. *CRC Crit Rev Biochem* 1984; **16**: 207–233.
- Pricer WE, Ashwell G. The binding of desialylated glycoproteins by plasma membranes of rat liver. *J Biol Chem* 1971; **246**: 4825–4833.
- Zanta MA, Boussif O, Adib A, Behr JP. In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. *Bioconjugate Chem* 1997; **8**: 839–844.
- Bettinger T, Remy JS, Erbacher P. Size reduction of galactosylated PEI/DNA complexes improves lectin-mediated gene transfer into hepatocytes. *Bioconjugate Chem* 1999; **10**: 558–561.
- Morimoto K, Nishikawa M, Kawakami S, *et al.* Molecular weight-dependent gene transfection activity of unmodified and galactosylated polyethyleneimine on hepatoma cells and mouse liver. *Mol Ther* 2003; **7**: 254–261.
- Sagara K, Kim SW. A new synthesis of galactose-poly(ethylene glycol)-polyethylenimine for gene delivery to hepatocytes. *J Control Release* 2002; **79**: 271–281.
- Nishikawa M, Takemura S, Takakura Y, Hashida M. Targeted delivery of plasmid DNA to hepatocytes in vivo: optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties. *J Pharmacol Exp Ther* 1998; **287**: 408–415.
- Choi YH, Liu F, Park JS, Kim SW. Lactose-poly(ethylene glycol)-grafted poly-L-lysine as hepatoma cell-targeted gene carrier. *Bioconjugate Chem* 1998; **9**: 708–718.
- Midoux P, Mendes C, Legrand A, *et al.* Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. *Nucleic Acids Res* 1993; **21**: 871–878.
- Han J, Yeom YI. Specific gene transfer mediated by galactosylated poly-L-lysine into hepatoma cells. *Int J Pharm* 2000; **202**: 151–160.

23. Niidome T, Urakawa M, Sato H, *et al.* Gene transfer into hepatoma cells mediated by galactose-modified alpha-helical peptides. *Biomaterials* 2000; **21**: 1811–1819.
24. Ferrari S, Moro E, Pettenazzo A, Behr JP, Zacchello F, Scarpa M. ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Ther* 1997; **4**: 1100–1106.
25. Pitard B, Bello-Roufai M, Lambert O, *et al.* Negatively charged self-assembling DNA/poloxamine nanospheres for in vivo gene transfer. *Nucleic Acids Res* 2004; **32**: e159.
26. Berry MN, Friend DS. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol* 1969; **43**: 506–520.
27. Balavoine S, Feldmann G, Lardeux B. Regulation of RNA degradation in cultured rat hepatocytes: effects of specific amino acids and insulin. *J Cell Physiol* 1993; **156**: 56–62.
28. Dentin R, Pégrier JP, Benhamed F, *et al.* Hepatic glucokinase is required for the synergistic action of ChREBP and SREBP-1c on glycolytic and lipogenic gene expression. *J Biol Chem* 2004; **279**: 20314–20326.
29. André C, Niamke S, Faure A, *et al.* New methods for chemo-enzymatic galactosidation of 2S rapeseed protein. *Protein J* 2004; **23**: 247–254.
30. Pitard B, Oudrhiri N, Lambert O, *et al.* Sterically stabilized BGTC-based lipoplexes: structural features and gene transfection into the mouse airways in vivo. *J Gene Med* 2001; **3**: 478–487.
31. Turek J, Dubertret C, Jaslin G, Antonakis K, Scherman D, Pitard B. Formulations which increase the size of lipoplexes prevent serum-associated inhibition of transfection. *J Gene Med* 2000; **2**: 32–40.
32. Yin C, Ying L, Zhang PC, *et al.* High density of immobilized galactose ligand enhances hepatocyte attachment and function. *J Biomed Mater Res A* 2003; **67**: 1093–1104.