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PEGylation potentiates hepatoma cell targeted liposome-mediated in vitro gene delivery via the asialoglycoprotein receptor

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Abstract: Hepatocellular carcinoma is a burgeoning health issue in sub-Saharan Africa and East Asia where it is most prevalent. The search for gene medicine treatment modalities for this condition represents a novel departure from current treatment options and is gaining momentum. Here we report on nonPEGylated and on sterically stabilized PEGylated cationic liposomes decorated with D-galacto moieties linked to 24.1 Å spacers for asialoglycoprotein receptor (ASGP-R)-targeted vehiculation of pCMV-luc plasmid DNA. Cargo DNA is fully liposome associated at N/P ratio = 3 : 1 and is partially protected from the effects of serum nucleases. Moreover, at this ratio, lipoplex dimensions (89–97 nm) are compatible with the requirements for extravasation in vivo. Ethidium displacement assays show that the reporter DNA is in a less condensed state when bound to PEGylated liposomes than with nonPEGylated liposomes. PEGylated lipoplexes were well tolerated by both HEK293 (ASGP-R-negative) and HepG2 (ASGP-R-positive) cell lines and delivered DNA to the human hepatoma cell line HepG2 by ASGP-R mediated at levels three-fold greater than nonPEGylated lipoplexes. PEGylated ASGP-R-targeted liposomes reported in

this study possess the required characteristics for hepatotropic gene delivery and may be considered for further application in vivo.

Keywords: asialoglycoprotein receptor; gene delivery; HepG2 cells; lipoplexes.

1 Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-associated deaths worldwide [1] and is particularly prevalent in sub-Saharan Africa and East Asia [2], where hepatitis B, its main cause, is endemic [3]. Indeed, >80% of HCC patients are seropositive for hepatitis B virus [4]. This condition is associated with a particularly poor prognosis, and current treatment options include surgical resection, liver transplantation, radiofrequency ablation, trans-arterial chemoembolization and systemic targeted sorafenib treatment [5, 6]. However, novel treatment modalities involving intervention at the genetic level are being actively investigated. These include an elegant gene therapy approach in which suicide genes are delivered to hepatoma cells by replicating virus vectors [7]. Such approaches also address the requirement for expression to be tumor-selective, as impaired liver function is often associated with HCC, and transgene expression should be limited to tumor cells alone without affecting normal hepatocytes [8]. Transcriptional targeting has therefore emerged as a promising approach in achieving this goal. Thus, tumor-specific α -fetoprotein enhancers [9] and core promoters [7] have been employed to drive expression of genes of interest. The high efficiency achieved by viral vectors is, however, often tempered by strong immune responses [10] and insertional mutagenesis [11]. This has given impetus to the further development of, as yet, less efficient but generally safer non-viral vectors [12]. Liposomal vehicles account for most of the non-viral vectors currently undergoing clinical trials worldwide [13], and their formulation flexibility, ease of manufacture and relatively low cost have ensured their ongoing popularity.

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The asialoglycoprotein receptor (ASGP-R), which is highly expressed on the basolateral side of the plasma membrane of hepatocytes and on hepatocyte-derived hepatoma cells and which displays high affinity for D-galactopyranosyl and N-acetylgalactosaminyl moieties [14–16], is a convenient target for the receptor-mediated uptake of lipoplexes carrying therapeutic genes [17]. The lipoplexes directed to the ASGP-R are internalized by clathrin-dependent receptor-mediated endocytosis into endosomes. The endosomal membrane anionic lipids interact with the cationic components of the lipoplexes forming charge-neutralized ion pairs, thereby displacing the cargo DNA and releasing it into the cytoplasm [18, 19]. Endosomal escape is also promoted by the helper lipid dioleoylphosphatidylethanolamine (DOPE) when present in lipoplexes in high concentration [18, 20]. The cone-shaped DOPE favors non-bilayer hexagonal phase formation, which destabilizes the endosomal membrane [21]. Although the ligand asialoorosomucoid, which displays D-galactopyranose units at the non-reducing ends of its heteroglycan appendages, has been tagged to liposomes for ASGP-R-mediated gene delivery [22, 23], several simpler constructs have emerged in which the galactopyranosyl moiety is affixed directly to one of the liposome components. Hence, liposomes that incorporate cholesteryl- β -D-galactopyranoside [24], lactosyl DOPE [25] and cholesteryl- β -N-(4-aminophenyl- β -D-galactopyranosyl) carbamate [26] all target DNA lipoplexes to the human HCC cell line HepG2. Transfection experiments conducted in vivo with cationic liposomes containing cholesteryl-5-yloxy-N-(4-((1-imino-2-D-thiogalactosylethyl)amino) butyl formamide (Gal-C4-Chol) reveal that lipoplexes measuring 141 nm in diameter achieve transfection levels in parenchymal cells significantly higher than in non-parenchymal cells [27]. In related studies siRNA lipoplexes decorated with galactopyranosyl units attached to the distal end of DSPE-PEG₂₀₀₀ have successfully directed siRNA to hepatocytes in vivo [28, 29]. Lipoplexes (liposome-DNA complexes) readily associate with serum proteins [30] and are rapidly marked for elimination by the reticuloendothelial system [31], thus severely limiting the availability of complexes at the intended target tissue. This may be mitigated, to a large extent, by anchoring poly(ethylene glycol)-2000 (PEG₂₀₀₀) to the liposomal membrane bilayer [32]. The biocompatible, hydrophilic, protective layer provided by PEG₂₀₀₀ limits opsonization and increases particle circulation times [31]. Nevertheless, lower transfection efficiencies are commonly observed with untargeted PEGylated lipoplexes. This PEG dilemma arises largely through reduced membrane fusion and limited non-bilayer intermediate formation [33].

In the present study we have prepared galactosylated cationic liposomes constituted with the cytofectin Chol-T, DOPE, cholest-5-en-3-yl-2-[2-(2-[4-(β -D-galactopyranosyl-1-oxymethyl)-1H-1,2,3-triazol-1-yl]ethoxy)ethoxy]ethoxy]ethylcarbamate (**1** Figure 1), with and without 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀). Studies conducted in the ASGP-R-positive human hepatoma cell line, HepG2, indicate that PEGylation enhances ASGP-R-mediated transfection by galactosylated lipoplexes, while in the ASGP-R-negative human embryo kidney cell line HEK293 [34], transfection levels were significantly lower.

2 Experimental

2.1 Materials

DOPE and bicinchoninic acid (BCA) assay reagents were purchased from the Sigma Chemical Company (St Louis, USA). The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 4-[(2-hydroxyethyl)-piperazinyl]ethanesulphonic acid (HEPES) and ethidium bromide (EtBr) were purchased from Merck, Darmstadt, Germany. The 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ammonium salt (DSPE-PEG₂₀₀₀) was purchased from Avanti Polar Lipids, Alabaster (Alabama, USA). Plasmid DNA (pCMV-luc) was obtained from Plasmid Factory (Bielefeld, Germany). Agarose was brought in from Bio-Rad Laboratories (California USA). HEK293 cell lines were donated by the anti-viral gene therapy Unit, University of the Witwatersrand, and HepG2 cell lines were obtained from Highveld Biological (PTY) LTD, Lyndhurst, South Africa. The luciferase assay kit was purchased from Promega Corporation (Madison, WI, USA). Minimum essential medium (MEM) containing Earle's salts and L-glutamine, penicillin (5000 U/mL)/streptomycin (5000 μ g/mL), trypsin-versene and fetal bovine serum (FBS) were purchased from Lonza-BioWhittaker (Walkersville, MD, USA). Tissue culture plastic ware was obtained from Corning Incorporated (New York, USA). The β [N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) was synthesized as described [23]. Cholest-5-en-3-yl-2-[2-(2-[4-(β -D-galactopyranosyl-1-oxymethyl)-1H-1,2,3-triazol-1-yl]ethoxy)ethoxy]ethoxy]ethylcarbamate (**1**) and Cholest-5-en-3-yl-2-[2-(2-[4-(2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl-1-oxymethyl)-1H-1,2,3-triazol-1-yl]ethoxy)ethoxy]ethoxy]ethylcarbamate (**2**) were prepared as reported elsewhere [24]. Deionized 18 MOhm water (Milli-Q50) was used throughout.

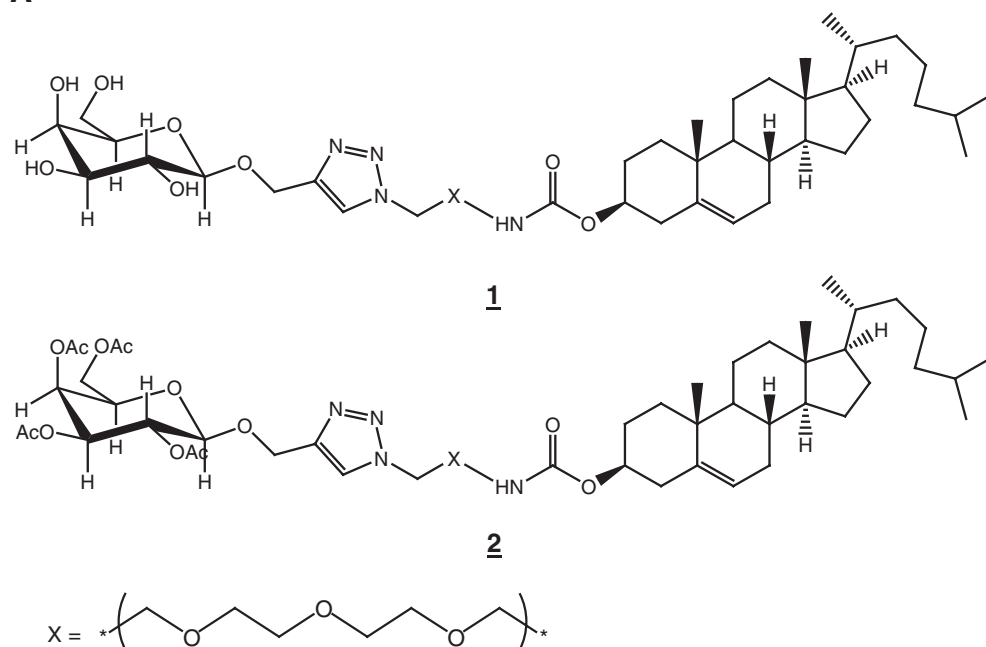
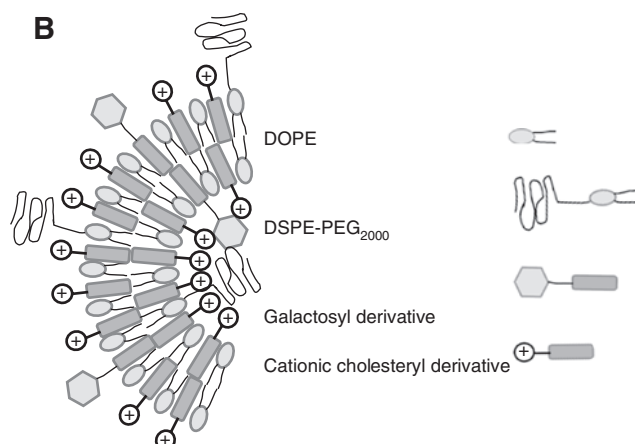
A**B**

Figure 1: (A) Galactopyranosyl cholesteryl derivatives **1**, cholest-5-en-3-yl-2-[2-(2-[4-(β-D-galactopyranosyl-1-oxymethyl)-1H-1,2,3-triazol-1-yl]ethoxy)ethoxy]ethoxy]ethylcarbamate; **2**, cholest-5-en-3-yl-2-[2-(2-[4-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-1-oxymethyl)-1H-1,2,3-triazol-1-yl]ethoxy)ethoxy]ethoxy]ethylcarbamate. (B) Schema of galactosylated, PEGylated cationic liposome membrane bilayer.

2.2 Liposome preparation

Liposomes were prepared by the thin film hydration method [35], to contain 4 μmol of total lipid in 1 mL of HEPES-buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.5). Briefly, individual components were dissolved in chloroform (10 μg/μL), except **1**, (pyridine) and combined in the ratios indicated in Table 1. This was then deposited as thin films in test tubes by rotary evaporation of solvent in vacuo (Büchi Rotavapor-R, Bern, Switzerland). Samples were dried under high vacuum in a drying pistol for 24 h.

Films were then rehydrated in sterile HBS overnight. Samples were vortexed (5 min) and sonicated for 5 min in a bath-type sonicator (Transsonic 460/H) at a frequency of 35 kHz to afford unilamellar liposomes.

2.3 Cryo-transmission electron microscopy of liposomes and lipoplexes

Samples were diluted in HBS (1:5, v/v) to promote fluidity of the samples. To aliquots (1 μL) of each diluted

Table 1: Liposome and lipoplex composition and dimensions.

Liposomes							Lipoplexes ^c	
Formulation	Chol-T ^a	DOPE ^a	Ligand ^a	DSPE-PEG ₂₀₀₀ ^a	Z average ^b (nm)	PDI	Z average ^b (nm)	PDI
1	2.0	1.6	0.4	–	144.4	0.216	96.7	0.203
1PEG	2.0	1.4	0.4	0.2	80.0	0.205	88.8	0.708
2	2.0	1.6	0.4	–	198.9	0.450	119.5	0.239
2PEG	2.0	1.4	0.4	0.2	105.1	0.227	110.7	0.222

^aMicromoles per mL of liposome dispersion in HBS. ^bIntensity weighted mean hydrodynamic diameter. ^cLipoplexes were assembled from liposomes and pCMV-luc plasmid DNA at N/P ($\pm$) ratio = 3 : 1. In calculating $\pm$ ratio, Chol-T is assumed to be fully protonated at pH 7.5, while DNA deoxyribonucleotide units are assumed to have a molecular weight of 330 Daltons and to carry 1 negative charge.

sample placed on Formvar coated grids was added uranyl acetate solution (2% w/v, 1 μ L). Excess liquid was blotted with filter paper. Thereafter, grids were plunged into liquid nitrogen cooled propane gas (-170°C) using a spring-loaded Leica CPC system. Grids were then viewed in a JEOL 1010 transmission electron microscope (TEM) (Tokyo, Japan) without warming above -150°C (accelerating voltage 100 kV). Images were captured using a Soft Imaging Systems (SIS) Mega View III digital camera with ITEM UIP software (Tokyo, Japan).

2.4 Particle sizing by dynamic light scattering

Hydrodynamic diameters of liposomes and lipoplexes were determined by a dynamic light scattering system using the photon correlation spectroscopy technique (ZetaSizer Nano-ZS, Malvern Instruments, Worcestershire, UK). Samples were equilibrated to room temperature for 15 min prior to measurement. Briefly, lipoplexes (containing 1 μ g of pCMV-luc) were prepared at desired N/P ratios (3 : 1). Following incubation, liposome (50 μ L) and lipoplex (10 μ L) suspensions were diluted to 1000 μ L with filter-sterilized HEPES buffered saline (HBS) and briefly vortexed prior to measurement at 25°C .

2.5 Gel retardation assay

Liposome-DNA complexes were formed by adding varying quantities of liposome dispersions to pCMV-luc plasmid DNA (0.5 μ g), and volumes were adjusted to 8 μ L with HBS. After incubation at room temperature (30 min), gel loading buffer (40% sucrose, 0.5% w/v bromophenol blue, 3 μ L) was added to samples before subjecting to electrophoresis on agarose 1% gels, containing 1 μ g/mL EtBr, in 36 mM Tris HCl, 30 mM NaH_2PO_4 , 10 mM EDTA

(ethylenediaminetetraacetic acid) and pH 7.5 buffer at 50 V for 90 min. Gels were then viewed by UV₃₀₀ transillumination, and images were captured using a Vacutec Syngene G: Box gel documentation system.

2.6 Serum nuclease protection assay

Lipoplexes were prepared and matured as described for the retardation assay. Thereafter, FBS was added to a final concentration of 10% v/v. Samples were further incubated at 37°C for 4 h following which, EDTA and sodium dodecyl sulphate were added to all samples to final concentrations of 10 mM and 0.5% (w/v), respectively. Mixtures were then incubated at 55°C for 20 min. Samples were then subjected to 1% agarose gel electrophoresis at 50 V for 120 min.

2.7 Ethidium bromide intercalation assay

The association and condensation of pCMV-luc plasmid DNA by targeted cationic liposomes was followed in an EtBr displacement assay. The assay is based on the observation that upon intercalation with double stranded DNA the planar polycyclic EtBr displays a marked increase in fluorescence with the quantum yield increasing by about 15-fold at 600 nm [36]. The introduction of increasing amounts of cationic liposomes partially displaces the dye with attendant quenching of fluorescence [37] until no further drop in fluorescence is observed [38]. Briefly, 2 μ L of stock ethidium bromide solution (1 mg/mL) was added to 100 μ L of HBS in a FluorTrac flat-bottomed black 96-well plate, and 0% baseline relative fluorescence was established at excitation and emission wavelengths of 520 and 600 nm in a Glomax[®]-Multi+ detection system (Promega). Thereafter, 2 μ L (0.5 μ g) of pCMV-luc DNA was added to the mixture, and the new reading was assumed to represent

100% relative fluorescence. Following this, 0.5 µg aliquots of the liposome preparations were added stepwise. Readings were taken 30 s after each addition until a plateau had been reached. Results obtained were plotted as relative fluorescence (F_r) percentages against mass of cationic liposome, where $F_r (\%) = (F_i - F_0) / (F_{\max} - F_0) \times 100$. Here F_0 is the fluorescence of ethidium bromide alone, while $F_{\max}$ is the fluorescence intensity of DNA fully intercalated with EtBr, and F_i is the fluorescence intensity at given concentrations of liposome.

2.8 Cell culture

Human embryonal kidney cells (HEK293) and human hepatoma cells (HepG2) were propagated in gas-permeable 25 cm² flasks in MEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in a humidified atmosphere containing 5% CO₂. Cells were divided 1 : 3 every 3–4 days.

2.9 Cytotoxicity studies

The cytotoxicities of lipoplexes at N/P (±) ratio of 3 : 1 were measured using the MTT assay, in which 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is reduced to form a colored insoluble formazan product by metabolically active cells [39]. HepG2 and HEK293 cells were trypsinized and seeded in 48-well plates at a density of 1.5×10^5 cells per well. Cells were then incubated in complete medium containing 10% FBS and antibiotics (300 µL) at 37 °C for 24 h. Lipoplexes were prepared by mixing pCMV-luc DNA (0.5 µg) with HBS and liposomes to a final volume of 10 µL, followed by a maturation period of 30 min at room temperature before use. Medium bathing cells was replaced with 300 µL of fresh, complete medium before addition of the lipoplexes. The assay was carried out in triplicate. Plates were then incubated at 37 °C for 48 h. After incubation, the medium was removed and cells were washed twice with 300 µL PBS. Thereafter, 300 µL of MTT solution (5 mg/mL in PBS) and 0.2 mL complete medium (10% FBS and MEM) were added to each well containing cells, and this was incubated for a further 4 h at 37 °C. MTT solution and medium were then removed and replaced with 300 µL of dimethylsulfoxide to extract the insoluble formazan crystals. Absorbances of extracts were measured at 570 nm using a Vacutec Mindray-MR-96A microplate reader. The percentage cell viability was calculated as follows: % cell survival = $[A_{570 \text{ nm}} \text{ treated cells}] / [A_{570 \text{ nm}} \text{ untreated cells}] \times 100$.

2.10 Transfection of human cell lines

HEK293 and HepG2 cells were plated into two separate 48-well plates, incubated overnight at 37 °C, and treated with lipoplexes as described for the cytotoxicity studies. Plates were incubated for 48 h at 37 °C. In competition assays, asialofetuin (200 µg) was introduced into wells 30 min before exposure to lipoplexes. After the incubation period, cells were assayed for luciferase activity using the Promega luciferase assay kit. Briefly, medium was removed from wells by aspiration, and cells were washed with phosphate buffered saline (2 × 100 µL). Thereafter, 80 µL of Promega cell lysis buffer was added to each well. The multiwell plates were placed on a platform rocker for 15 min at 30 revolutions/min. Cell lysates were transferred into microcentrifuge tubes (1.5 mL) followed by centrifugation at $12,000 \times g$ for 5 s to pellet cell debris. To 20 µL aliquots of clear supernatants were added 100 µL of Promega luciferase assay reagent at room temperature. After brief agitation, luminescence was measured in a Lumac Bio-counter 1500 luminometer. Protein concentrations of the cell-free extracts were determined using the BCA assay with bovine serum albumin (BSA) as standard. Luciferase activity was expressed as the relative light units/mg of protein (RLU/mg protein).

2.11 Statistical analysis

GraphPad Prism 5 (USA) was used to perform statistical analysis of the results using the t-test, where $P < 0.05$ was considered to indicate a significant difference.

3 Results and discussion

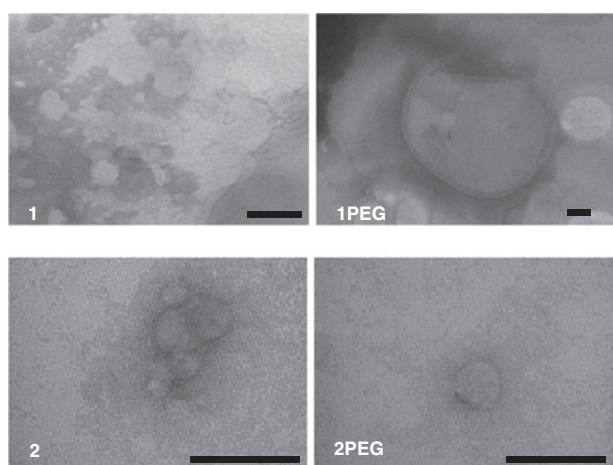
The galactopyranosyl cholesteryl derivative cholest-5-en-3-yl-2-[2-(2-[4-(β-D-galactopyranosyl-1-oxymethyl)-1H-1,2,3-triazol-1-yl]ethoxy)ethoxy]ethylcarbamate (**1**, Figure 1), which incorporates a hydrophilic 24.1 Å spacer element separating the sugar component from the sterol, was obtained by the deacetylation of its peracetylated precursor cholest-5-en-3-yl-2-[2-(2-[4-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-1-oxymethyl)-1H-1,2,3-triazol-1-yl]ethoxy)ethoxy]ethylcarbamate (**2**, Figure 1) with K₂CO₃ in methanol. This, in turn, had been synthesized by an alkyne-azide cycloaddition (CuAAC) ‘click’ reaction between the peracetylated galacto alkyne, 2,3,4,6-tetra-O-acetyl-1-(2'-propargyl)-β-D-galactopyranose, and cholest-5-en-3-yl-2-[2-(2-(2-azidoethoxy)ethoxy)ethoxy]ethylcarbamate.

Liposomes were formulated to contain 10 mol% of **1** or **2** with or without 5 mol% DSPE-PEG₂₀₀₀ (Table 1) and exhibited hydrodynamic diameters in the 80–200 nm range (Table 1 and Figure 2). PEGylated liposomes were almost 50% smaller than their nonPEGylated counterparts due to inter-bilayer repulsion induced by PEG on the liposome surface [40]. Cryo-TEM revealed that liposome preparations comprised spherical and ellipsoidal unilamellar vesicles (Figure 2). PEG₂₀₀₀ was selected for this study over larger or smaller molecular sizes of PEG to achieve longer circulation times and a balance between steric hindrance to prevent opsonization [41] and permitting ligand-receptor interaction for receptor-mediated uptake of lipoplexes. Inclusion in liposome formulations at 5 mol% was to ensure the formation of an effective steric barrier yet avoiding membrane disruption and possible

micelle formation, which may occur at higher PEG concentrations [42–44]. Moreover, at 5 mol% PEG, chains are largely arranged in a ‘brush regime’, which affords thermodynamic stability to liposomes [45] and resistance to clearance by macrophages [46]. The galactosyl ligand was incorporated at the 10 mol% level to favor lipoplex interaction with the hepatocytic ASGP-R [19] over binding to a similar C-type lectin located on scavenger nonparenchymal Kupffer cells [47] of the liver, which is known to occur at higher galactosyl grafting densities [48].

The association between liposomes and plasmid DNA was followed by two separate and independent assays. Thus, in gel retardation assays, plasmid DNA was fully liposome-associated at N/P ratio = 3:1 (Figure 3A–D), while in ethidium displacement experiments, cargo DNA was maximally condensed at N/P ratios = 2:1–3:1 (Figure 4). Results also revealed that plasmid DNA was condensed to a higher degree by unPEGylated liposomes **1** and **2**, with 70% ethidium displacement, than by PEGylated liposomes, which was accompanied by a 50% ethidium

Liposomes



Lipoplexes

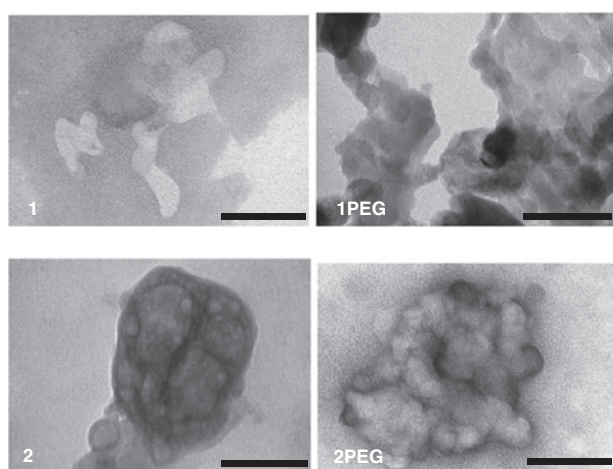


Figure 2: Cryo-transmission electron micrographs of liposomes and lipoplexes. Scale bar = 100 nm.

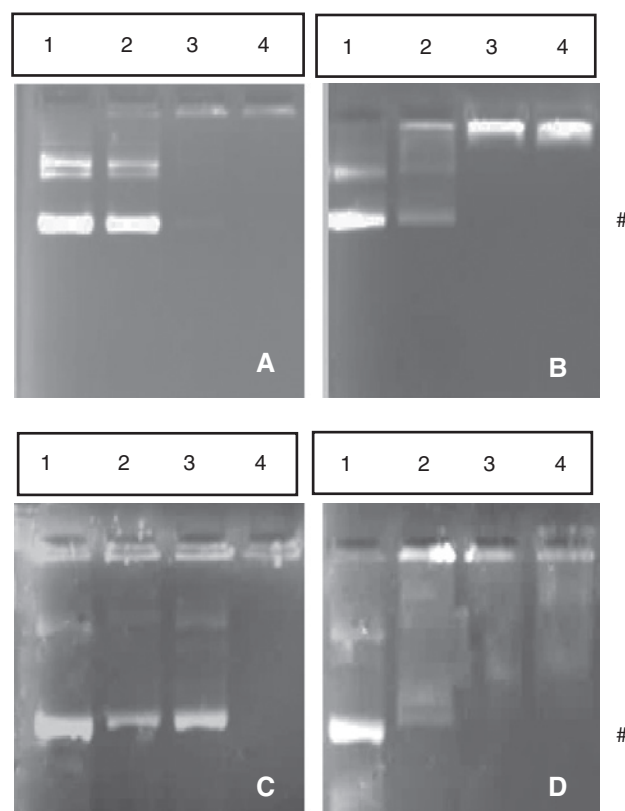


Figure 3: Gel retardation assay of binding between liposomes and plasmid DNA. Incubation mixtures in HBS (8 μ L) contained pCMV-luc plasmid DNA (0.5 μ g) and varying amounts of liposomes to achieve N/P ratios + 1:1, 2:1 and 3:1 in lanes 2–4, respectively. Lane 1 contained plasmid DNA alone. (A) Liposomes **1**, (B) **1PEG**, (C) **2**, and (D) **2PEG**. Superhelical DNA is indicated (#).

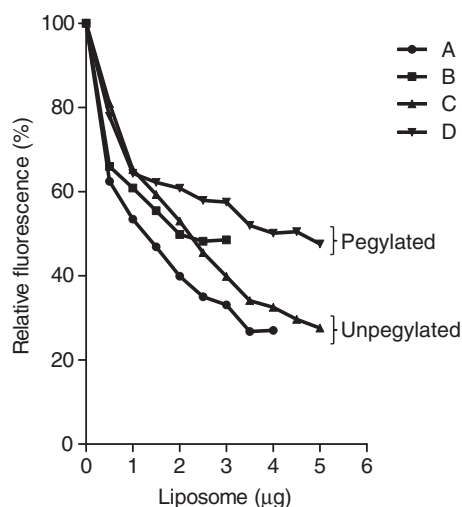


Figure 4: Ethidium bromide intercalation assay. Incubation mixtures in HBS (104 μ L) containing EtBr (2 μ g) and pCMV-luc plasmid DNA (0.5 μ g) were treated with increasing amounts of liposome suspension (0.5 μ g aliquots).

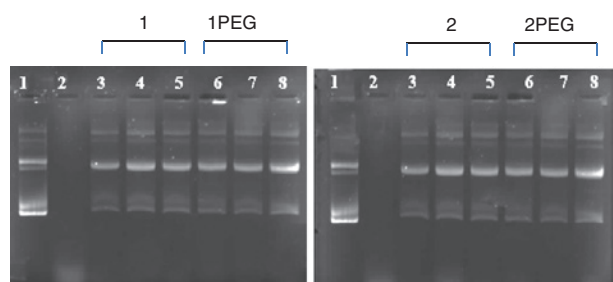


Figure 5: Serum nuclease protection assay of lipoplex-associated DNA. Mixtures (10 μ L) contained pCMV-luc DNA (0.5 μ g), liposomes (N/P ratios + 1:1, 2:1 and 3:1 in lanes 3–5 and 6–8) and FBS (10%, v/v) in HBS. Lane 1: untreated plasmid DNA; lane 2: plasmid DNA in the presence of FBS. Relaxed closed circular (*) and superhelical (#) plasmid DNA are indicated.

displacement (Figure 4). The integrity of liposome-bound plasmid DNA in the presence of HBS was monitored by agarose gel electrophoresis. All liposomes offered partial protection to the plasmid DNA in the N/P range 1:1–3:1 (Figure 5). Gels revealed that plasmid DNA was largely in the nicked, relaxed closed circular form after exposure to HBS. This topological form of pCMV-luc DNA has been shown to yield higher transfection activities than either the superhelical or linear conformers when delivered by cationic liposomes to cells in vitro [49]. It is also likely that superhelical DNA may be nicked during its intracellular journey to the nucleus [50].

Cell viability studies after exposure to liposome-DNA complexes under transfection conditions (N/P = 3:1) were conducted in HEK293 (ASGP-R-negative) and in

HepG2 (ASGP-R-positive) cell lines. Results indicate that PEGylated lipoplexes were better tolerated (>70% viability) in both cell lines than nonPEGylated complexes (50–60% viability) (Figure 6). Transfection experiments revealed that both lipoplexes 1 and 1PEG achieved higher transfection activity in HepG2 cells than in the HEK293 cell line, with 1PEG transfection levels approximately 3 $\times$ higher than those achieved by lipoplex 1, while transfection

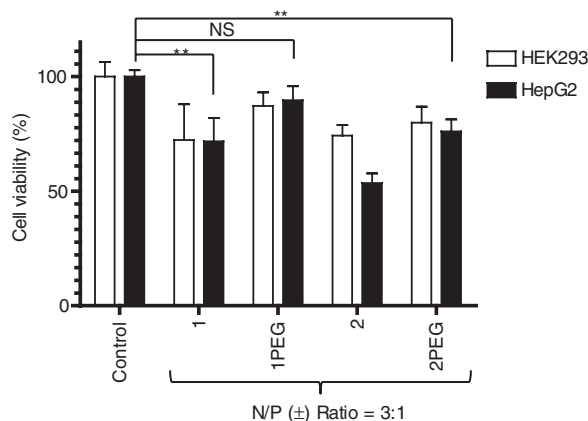


Figure 6: Cytotoxicity assay of lipoplexes in HEK293 and HepG2 cells. Cells in 48-well plates bathed in 300 μ L of complete medium containing antibiotics and FBS (10%) were treated with lipoplexes at N/P ratio = 3:1 (0.5 μ g plasmid). Data are presented as means $\pm$ SD (n = 3). ** P < 0.01 for indicated comparisons.

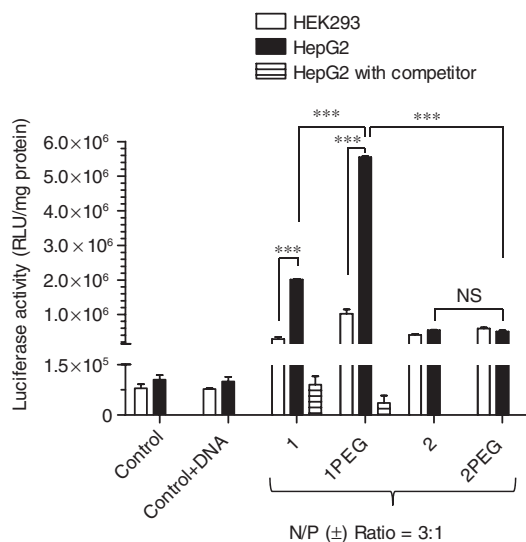


Figure 7: Transfection studies of lipoplexes in HEK293 and HepG2 cells. Cells bathed in complete medium (300 μ L, 48-well plates) were exposed to lipoplexes (N/P ratio = 3:1) containing 0.5 μ g pCMV-luc plasmid DNA. Control: untreated cells; Control + DNA: cells exposed to naked plasmid DNA (0.5 μ g). In competition assays, wells contained 200 μ g asialofetuin. Data are presented as means $\pm$ SD (n = 3). Differences between 1/1PEG and 1PEG/2PEG are statistically very significant (** P < 0.001).

activities in the presence of the ASGP-R ligand, asialofetuin, were reduced to background levels. Lipoplexes 2 and 2PEG were less effective with no discernible differences between the two cell lines (Figure 7). This may be attributed largely to the acylated sugar hydroxyl groups, which may prevent correct binding of the galacto moiety to the ASGP-R. It has been shown that the specificity of ASGP-R for terminal D-galacto residues results from H-bonding between the galacto 3-OH and 4-OH groups with carboxylate and amide side chains in the ASGP-R binding site [17]. High transfection levels achieved by PEGylated lipoplex 1PEG in HepG2 cells may be attributable, in part, to the looser association of the cargo DNA, which may therefore be more readily released upon cell entry of the lipoplex by receptor mediation.

4 Conclusion

We report on PEGylated and nonPEGylated liposomes decorated with D-galacto and peracetylated D-galacto residues appended to 24.1 Å spacer elements and their vehiculation of pCMV-luc plasmid DNA in transfection experiments *in vitro*. Results confirm that PEGylated galacto liposomes very effectively direct reporter DNA to the human hepatoma cell line HepG2 largely by ASGP-R mediation. Physicochemical studies also indicate that lipoplexes fall within an acceptable size range for extravasation *in vivo* and that DNA is adequately protected from serum nucleases. Moreover, DNA appears to be more tenuously associated with PEGylated liposomes than with their unPEGylated counterparts. In summary, PEGylated ASGP-R-targeted liposomes reported herein possess the required characteristics for hepatotropic gene delivery and may be considered for further development in an *in vivo* environment.

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