Targeting of glycosylated lipoplexes in HepG2 cells: Anomeric and C-4 epimeric preference of the asialoglycoprotein receptor

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This study was conducted to determine the capacity of the asialoglycoprotein receptor on the hepatocyte-derived cell line HepG2 to exhibit an anomeric preference with respect to the D-galacto moiety on cationic liposome membrane-anchored cholesteryl-α-D-galactopyranoside (Choαgal) and cholesteryl-β-D-galactopyranoside (Cholβgal) in cationic liposome/pGL3 plasmid DNA complexes constructed for non-viral, hepatocyte-directed gene transfer. In addition, cholesteryl-α-D-glucopyranoside (Choαglu) and cholesteryl-β-D-glucopyranoside (Cholβglu) were separately formulated into cationic liposomes at the same mole ratio (11%) to examine the C-4 epimeric selectivity of the asialoglycoprotein lectin for the glucopyranosides displayed in derived lipoplexes. Lipoplex formation was examined by gel retardation, ethidium displacement assays, and transmission electron microscopy. Plasmid DNA was shown to be fully liposome associated and maximally compacted at a liposome:DNA ratio of 6:1 (weight ratio), corresponding to a +/– charge ratio of 1.3 with complexes falling in the 80–200 nm size range, whereas at a 5:1 w/w ratio [1.1 (+/–) charge ratio] lipoplexes were somewhat smaller (50–100 nm) but promoted higher transgene activity in HepG2 cells than 6:1 (w/w) lipoplexes, in the following order: Cholβgal > Cholαgal > Cholβglu = Cholαglu. Transgene activity levels in HeLa cells which lack the asialoglycoprotein receptor were approximately 10% of those achieved in HepG2 cells. Moreover, transgene activity in HepG2 cells was reduced by approximately 90% in the presence of excess asialofetuin, a ligand for the asialoglycoprotein lectin.

Introduction

Among the non-viral approaches being investigated for the cell-specific delivery of corrective genes *in vitro* and *in vivo* into mammalian cells, those designed for delivery to liver parenchymal cells are arguably the best characterized. The asialoglycoprotein receptor (ASG-R), an endocytic recycling receptor which is highly represented on the sinusoidal face of the hepatocyte plasma membrane,[1] is the lectin of preference for the modulation of cell-entry by variously vehiculated DNA constructs. The importance of these endeavours is underscored by the fact that malfunction of the liver often leads to serious metabolic damage affecting the entire body. The receptor-mediated delivery of DNA into hepatocytes is frequently studied *in vitro* in the hepatocyte-derived human hepatocellular carcinoma cell line HepG2, which is known to express the ASG-R[2] and has the same biosynthetic capabilities as normal liver parenchymal cells.[3] Since Wu and Wu transfected, by receptor mediation, first HepG2 cells[4] and subsequently rat liver[5] with DNA complexed to asialoorosomucoid (AOM) and poly-L-lysine, AOM-derived hepatocyte-specific DNA delivery systems have been widely studied.[6–9]

The complexity associated with the assembly of glycoprotein-based delivery systems has provided the impetus for the development of simpler constructs. In several alternative hepatocyte-specific DNA delivery systems, some of which are listed below, the D-galactose moiety located at each non-reducing terminus of the triantennary N-linked heteroglycan structures on asialoorosomucoid, which is required for ASG-R recognition, has been appended to: albumin,[10] histones,[11] α-helical peptides,[12] polylysine,[13–15] lysine-serine co-polymers,[16] polyethyleneimine,[17] polypropyleneimine dendrimers,[18] lipopolyamine,[19] chitosan,[20] LDL and HDL[21] and cationic liposomes.[22,23]

D-galactopyranosyl moieties in simple glycosidic link with cholesterol, a component of animal cell membranes, have not hitherto been formulated into liposomes destined for hepatocyte delivery of DNA. The specificity of the ASG-R for the terminal D-galactosyl moieties of glycoproteins is achieved through hydrogen bonding of the sugar 3- and 4-hydroxyl groups with receptor carboxylate and amide side chains.[24] D-glucose, an epimer of D-galactose, which differs from this sugar in the configuration of the 4-hydroxyl group (equatorial), would therefore be expected to be more poorly recognized by the ASG-R. Moreover, terminal D-galactosyl moieties on asialoorosomucoid are in a β-glycosidic linkage. This anomeric configuration has also been retained in neoglycoproteins,[10,11] peptides,[12–16] and other constructs exhibiting D-galactose for ASG-R targeting.[19–21]

In the study reported here, we prepared the α- and β-anomers of cholesteryl-D-galactopyranoside and cholesteryl-D-glucopyranoside and incorporated them into cationic unilamellar liposomes. Subsequently, lipoplexes were assembled with pGL3 vector DNA and the anomeric and epimeric preferences of the ASG-R on HepG2 cells for sugar moieties displayed on lipoplexes (Fig. 1) was examined by measuring transfection levels attained in each case in the presence or absence of the competing ligand, asialofetuin. Cholesteryl-β-D-galactopyranoside-containing lipoplexes were convincingly more effective than the other glycosylated lipoplexes studied. It is noteworthy that the ASG-R appeared able to discern epimeric and anomeric features of the glycopyranosyl units displayed on lipoplex membrane bilayers, even when in direct glycosidic linkage to the cholesteryl anchor and therefore without an extensive spacer. Luciferase activities achieved by all four lipoplexes in HeLa cells, which are ASG-R-negative, were very low.

Materials and methods

Chemicals and spectrometry

Dioleoylphosphatidyl ethanolamine (DOPE) was from Sigma (St Louis, MI). β-D-glucose pentaacetate was from Aldrich (Milwaukee, WI) and α, β-D-galactose pentaacetate was from Pfanstiehl (Waukegan, IL). 2-[-(2-hydroxyethyl)-piperazinyl]-ethanesulphonic acid (HEPES) and cadmium carbonate were from Merck (Darmstadt, Germany). Trypsin-

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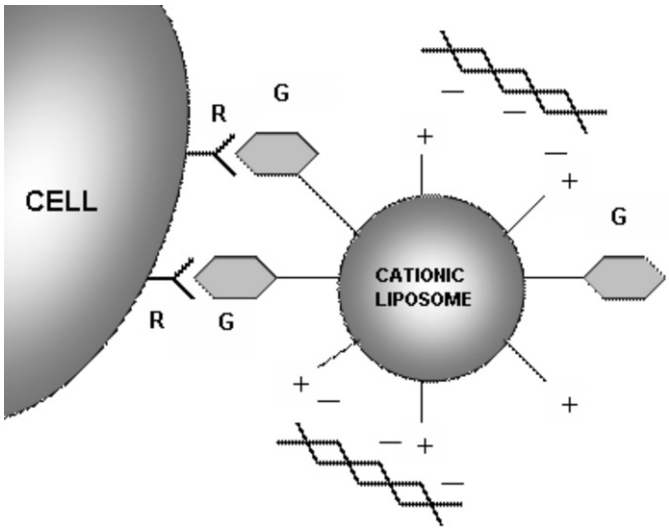


Fig. 1. The galactosylated cationic liposome interaction with its DNA cargo and the accommodation of this assembly by the plasma membrane-bound asialoorosomucoid receptor (ASG-R). R = ASG-R; G = glycopyranosyl moiety. Figure not to scale.

EDTA and penicillin-streptomycin mixtures were supplied by Whittaker Bioproducts (Walkerville, MD). N,N-dimethylaminopropylamidocholesterylformylhydrazide (MS09) was prepared as reported.[25] ^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded at 300 MHz and at 75 MHz, respectively, on a Varian Gemini 300 instrument. Chemical shifts (δ) were recorded relative to $CDCl_3$ (7.24 ppm) or C_5D_5N (8.57 ppm) for ^{1}H and $CDCl_3$ (77 ppm) or C_5D_5N (135.5 ppm) for ^{13}C. Signal multiplicities are abbreviated as follows: s(singlet), d(doublet), t(triplet) and m(multiplet). Chemical shifts are reflected as parts per million (ppm) and are listed for ^{1}H spectra as: shifts (multiplicity, integration, coupling, assignment). Spectral data for cholesteryl-tetra-*O*-acetyl glycosylpyranosides and cholesteryl-α (and β)-D-glycopyranosides appear in supplementary material online.

Syntheses

*Cholesteryl-tetra-*O*-acetyl glycosylpyranosides*. Cholesteryl-tetra-*O*-acetyl glycopyranosides were prepared by a modified Koenigs–Knorr procedure from the respective tetra-*O*-acetyl glycopyranosyl bromide and cholesterol in the presence of cadmium carbonate[26] followed by deacetylation. Thus, tetra-*O*-acetyl glycopyranosyl bromides were prepared by passing HBr through a solution of D-glucopyranosyl pentaacetate or D-galactopyranosyl pentaacetate (7.8 g, 20 mmol) in glacial acetic acid (100 ml, 4°C). Completion of bromination was confirmed by TLC on silica gel $60F_{254}$ plates (Merck, Darmstadt) in hexane:ethyl acetate (8:3 v/v) followed by visualization using a *p*-anisaldehyde spray (*p*-anisaldehyde:conc. H_2SO_4: ethanol, 5:5:90 volume ratios). The solution was poured onto crushed ice and the bromo sugar was extracted into dichloromethane (130 ml), which was subsequently washed with a 1% sodium bicarbonate solution (100 ml) and water (2 × 200 ml). After drying over $CaCl_2$, solvent was removed *in vacuo* and the residue co-evaporated with toluene. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide or 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (20 mmol) in toluene (30 ml) was added dropwise to a refluxing solution of cholesterol (3.9 g, 10 mmol) in toluene (75 ml) over $CdCO_3$ (4 g, 22.7 mmol) in an apparatus fitted with a Dean–Stark trap. The reaction mixture was heated under azeotropic conditions for a further 2 hours, or until complete glycosylation was confirmed by TLC (method described above). The hot solution was filtered under vacuum (celite, Hartley funnel) to remove the inorganic salts. Solvent was removed by evaporation *in vacuo* and the residue dissolved in hexane:ethyl acetate (8:3 v/v, 30 ml). TLC revealed one major product (β-anomer) and a minor product (α-anomer). Anomers were separated on a silica gel 60 column (70–230 mesh, Merck), which was equilibrated and eluted with hexane: ethyl acetate (8:3 v/v). Finally, the cholesteryl tetra-*O*-acetyl glycosylpyranosides were recrystallized from ethanol.

*Cholesteryl-α(and β)-*D*-glycopyranosides.* The acetylated cholesteryl glycosides in $CHCl_3$ were treated with excess sodium ethoxide in ethanol for 8 hours at room temperature. Solvent was removed by rotary evaporation (25°C) and the residues triturated with water at 4°C. The powdery precipitates were resuspended in water, filtered and dried *in vacuo* at 60°C (Büchii-TO pistol drier).

Plasmid DNA

In this study, the pGL3 control vector (5256 bp), which encodes the *Photinus pyralis luciferase* gene flanked by SV40 promoter and enhancer sequences, was used (Promega, Madison, WI). This arrangement affords strong expression in a variety of mammalian cell types. The pBR322 plasmid (4363 bp) (Roche Diagnostics, Mannheim) was used in the dye displacement assay only.

Liposome preparation

Individual cholesteryl glycosides (0.5 μmol) were dissolved in pyridine (200 μl) and added to DOPE (2 μmol) and MS09 (2 μmol) in $CHCl_3$ (1.0 ml). Solvent was evaporated *in vacuo* and components deposited as a thin film. This was dried further for 2 hours under vacuum and the film was re-hydrated overnight in HBS (20 mM HEPES, 150 mM NaCl, pH 7.5, 1 ml). The re-hydration mixture was vortexed briefly and sonicated for 5 minutes (Transsonic bath-type sonicator). Preparations were routinely stored at 4°C for several weeks with no evidence of aggregation or precipitation.

Transmission electron microscopy

Size and lamellarity of liposomes were determined by TEM. Thus liposome suspensions (50 μl, 4.5 μmol lipid/ml HBS) on parafilm were mixed with 0.5% uranyl acetate (50 μl) and left to stand for 3 min, whereupon the matt surfaces of formvar-coated copper grids were brought into contact with the mixtures for 3 min. Thereafter, discs were air-dried overnight and viewed on a JEM-1010 transmission electron microscope (JEOL, Tokyo) operating at 60 kV. Images were photographed at a 2-second exposure on fine-grain release positive film. Lipoplexes formed by the addition of liposomes to plasmid DNA at ratios of 5:1 and 6:1 (w/w in HBS) were treated and viewed as described for liposomes.

Liposome–DNA interactions

Gel retardation assays. Liposome:DNA mixtures were prepared in HBS (10 μl) and incubated at 20°C for 30 min before mixing with 3 μl gel loading buffer (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol, 72 mM Tris-HCl, 60 mM NaH_2PO_4 and 20 mM EDTA at pH 7.5). Samples were loaded onto 1% agarose gels in a Mini-Sub® apparatus (Bio-Rad, Richmond, CA). Electrophoresis was carried out for 90 min at 50 V (Consort E455 power supply, Turnhout, Belgium) in TPE running buffer (36 mM Tris-HCl, 30 mM NaH_2PO_4, 10 mM EDTA, pH 7.5). Gels were treated for 30 min with ethidium bromide (1 μg/ml running buffer) and viewed under transillumination at 300 nm. Images were captured on a Gene Genius Bioimaging System (Syngene, Cambridge, U.K.).

Nuclease digestion assays. Liposome:DNA complexes were assembled from glycosylated cationic liposomes and pGL3 DNA in HBS (20 μl) and incubated for 30 min (20°C). Fetal bovine serum was added to a final concentration of 10% and samples were incubated at 37°C for 4 hours, whereupon SDS and EDTA were introduced to a final concentration of 0.5% (w/v) and 10 mM, respectively. Mixtures were then maintained at 55°C for 20 min before adding gel loading buffer (3 μl) and subjecting to agarose gel electrophoresis as described above.

Ethidium displacement assays. The association of liposomes with plasmid DNA to form lipoplexes was also studied in ethidium bromide (EtBr) displacement assays, which are based on the premise that on intercalation with DNA, the dye exhibits an increase of the fluorescence quantum yield[28] and on condensation of the nucleic acid the dye is displaced with a resultant decrease in fluorescence.[29] The assays performed in this study were adapted from that described by Tros de Ilardaya *et al.*[30] Fluorescence measurements were recorded on a Shimadzu RF-551 spectrofluorometric detector (Kyoto, Japan) at excitation and emission wavelengths of 520 and 600 nm, respectively. Briefly, baseline fluorescence (0%) was established with an EtBr solution in HBS (1 μg/500 μl buffer). Thereafter, pBR322 DNA (6 μg) was introduced and the solution was used to set the instrument to 100% fluorescence. Aliquots (3 μg) of glycosylated liposomes were then introduced and readings taken after mixing at each step, until 45 μg of glycosylated liposome had been added.

Cell culture

The human cervical carcinoma cell line HeLa and human hepatocellular carcinoma line HepG2 were from Highveld Biological, Kelvin, South Africa, and cultured in 25-cm^2 flasks in 5 ml of MEM (minimum essential medium) with Earle's salts (Gibco, BRL, Inchinnan, Scotland) containing 10% (v/v) FBS (Delta Bioproducts, Johannesburg), 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate (Whittaker, M.A. Bioproducts, MD), 10 mM $NaHCO_3$ and HEPES (pH 7.5). Cells were passaged 1:3 (HeLa) and 1:2 (HepG2) every 3–4 days and stored at –80°C in complete medium containing 10% DMSO (v/v).

Cell growth inhibition assays

Cells were trypsinized and seeded into 24-well plates (Bibby-Sterilin, Staffordshire, U.K.) at a density of 2×10^4 cells per well. These were incubated at 37°C for 24 hours in complete medium (0.5 ml) to allow cell attachment and growth to semi-confluency. Liposome–DNA complexes were formed in HBS (15 μl) and incubated at 20°C for 30 min. Growth medium bathing cells was removed and replaced by serum-free medium (0.5 ml) and lipoplexes were added. After incubation for 4 hours (37°C), the medium was replaced with complete medium and cells were incubated for an additional 48 hours. Adherent cells were quantified by the method of Schellekens and Stitz.[31] Briefly, cells were washed with PBS (2×1 ml) and stained with 200 μl crystal violet solution (0.8%, w/v sodium chloride; 5% v/v formaldehyde; 0.5% crystal violet and 50% v/v ethanol) for 20 min. After removal of stain, wells were washed exhaustively with water and plates were dried at room temperature (24 hours). Stain from cells was extracted into 2-methoxyethanol (500 μl, 36 hours) and absorbances were determined at 550 nm (Novaspec spectrophotometer, Biochrom, Cambridge, U.K.).

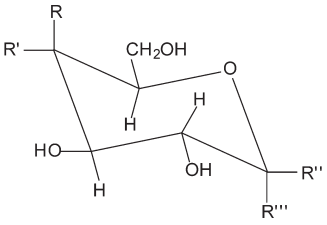
Transfection *in vitro*

Human hepatocellular carcinoma cells (HepG2) and cervical carcinoma cells (HeLa) were seeded into 24-well plates 24 hours prior to transfection, at a density of 2×10^4 cells per well in complete medium (0.5 ml). Lipoplexes containing glycosylated cationic liposomes (0–7 μg per well) and pGL3 plasmid DNA (1 μg per well) were prepared in 20 mM Hepes, 150 mM NaCl (15 μl per well) and incubated at 21°C for 30 min prior to transfection. Growth medium was removed from cells which were then washed with PBS (0.5 ml) and bathed in serum-free medium (MEM + antibiotics, 0.5 ml). Lipoplexes were added and after 4 hours at 37°C the medium was removed and replaced with complete medium (MEM + antibiotics + 10% FBS). After 48 hours cells were lysed and supernatants assayed for luciferase activity (Promega, Madison, WI) in a Lumac Biocounter M1500 (Landgraaf, Netherlands). Soluble protein in cell lysates was determined by the BCA assay (Sigma) using BSA as the protein standard. Transfection activities of all four liposome:DNA complexes in HepG2 cells were also evaluated in the presence of 10% FBS and separately in the presence of asialofetuin (300 μg per well).

Results and discussion

Cholesteryl glycosides

Four cholesteryl glycosides (Fig. 2) were synthesized for a study of their asialoglycoprotein receptor-targeting potential when incorporated into the bilayer of cationic liposomes. The syntheses were effected by a Koenigs–Knorr procedure from the appropriately protected bromo-sugar and cholesterol under anhydrous conditions.[26] During the course of this project, Wimmer and co-workers[32] reported a similar procedure for the preparation of glucoside and galactoside derivatives of cyclic (secondary) alcohols in good yield using cadmium carbonate as promoter under anhydrous conditions. Structures were confirmed by reference to ^{1}H and ^{13}C NMR spectra recorded in $CDCl_3$ for the intermediate cholesteryl-tetra-*O*-acetylglycosylpyranosides and in C_5D_5N (deuteropyridine) for the deprotected final products. ^{13}C signal assignments for the cholesteryl moieties were very similar to one another in the eight cholesteryl glycosides generated in the present study and are in agreement with assignments reported previously in an investigation of glycosidation shifts in ^{13}C NMR spectra of related



	R	R'	R''	R'''
Cholαgal	OH	H	H	Chol
Cholβgal	OH	H	Chol	H
Cholαglu	H	OH	H	Chol
Cholβglu	H	OH	Chol	H

Fig. 2. Cholesteryl glycosides. Cholαgal, cholesteryl-α-D-galactopyranoside; Cholβgal, cholesteryl-β-D-galactopyranoside; Cholαglu, cholesteryl-α-D-glucopyranoside; Cholβglu, cholesteryl-β-D-glucopyranoside.

sterylglycosides.[27] Chemical shifts of the carbohydrate components in the ^{1}H and ^{13}C NMR spectra of the compounds analysed agreed with those reported by Agrawal[33] (^{1}H) and Seo *et al*.[27] (^{13}C). ^{1}H peak assignments for the tetra-*O*-acetyl-β-D-galactoside entity were virtually identical to those reported by Ren *et al*.[34] in a study of galactosylated dendrimers containing multiple primary amino groups.

Liposome characterization by TEM

Liposomes prepared here were constituted from MS09, DOPE and cholesteryl glycosides (4:4:1 mole ratios, respectively) with the glycoside component limited to 9% on a weight ratio basis. Other ratios were not explored. It is apparent, however, that the sugar density in liposomes is an important factor. It was observed that at a ratio of 5% (w/w) liposome/glycolipid particles are efficiently processed by the ASG-R, whereas at a loading ratio of 50% (w/w) uptake is not blocked by excess asialofetuin.[35] It has also been demonstrated that in the loading range 0–7.5% (w/w) for the galactosyl steryl derivative Gal-C4-chol, the highest uptake of galactosylated liposomes by parenchymal cells was achieved at 5%.[36] A large number of galactose or glucose residues could also interfere with the electrostatic interaction of the liposomes with DNA. Liposomes prepared in this study are bifunctional, with each liposome possessing a cationic component (MS09) for binding to the DNA molecule and a terminal galactose or glucose component for targeting to the galactose or asialoglycoprotein receptor on the HepG2 cells. The co-lipid DOPE was chosen for its known ability to improve intracellular trafficking of lipoplexes[37] and for improving the rate of endosomal escape of the DNA cargo.[38] All liposome formulations were stable at 4°C for several weeks and showed no aggregation or precipitation of components. Transmission electron microscopy revealed the formation of predominantly spherical or oval vesicles and confirmed the unilamellar nature of all liposome preparations. Liposome Cholαgal sizes varied from 100 nm to 200 nm, liposome Cholβgal from 50 nm to 150 nm, liposome Cholαglu from 50 nm to 200 nm, and Cholβglu from 100 to 200 nm (see Fig. A(a)–(d) in online supplement, respectively).

Liposome–DNA interactions

To determine the optimal ratio for binding between liposomes and DNA, gel retardation assays are commonly performed. In Fig. 3(A–D) it can be seen that for all four liposomes no migration of the plasmid DNA was obtained at the liposome:DNA ratio of 6:1 (w/w), equivalent to a (+/–) charge ratio of 1.3:1. At this ratio and higher, all DNA was liposome-associated. In the ethidium displacement assays, liposomes which have been formulated to contain the synthesized glycolipids, readily bring about displacement of the intercalated ethidium bromide upon their stepwise introduction to the plasmid DNA. This process is largely driven by the association of DNA phosphodiester

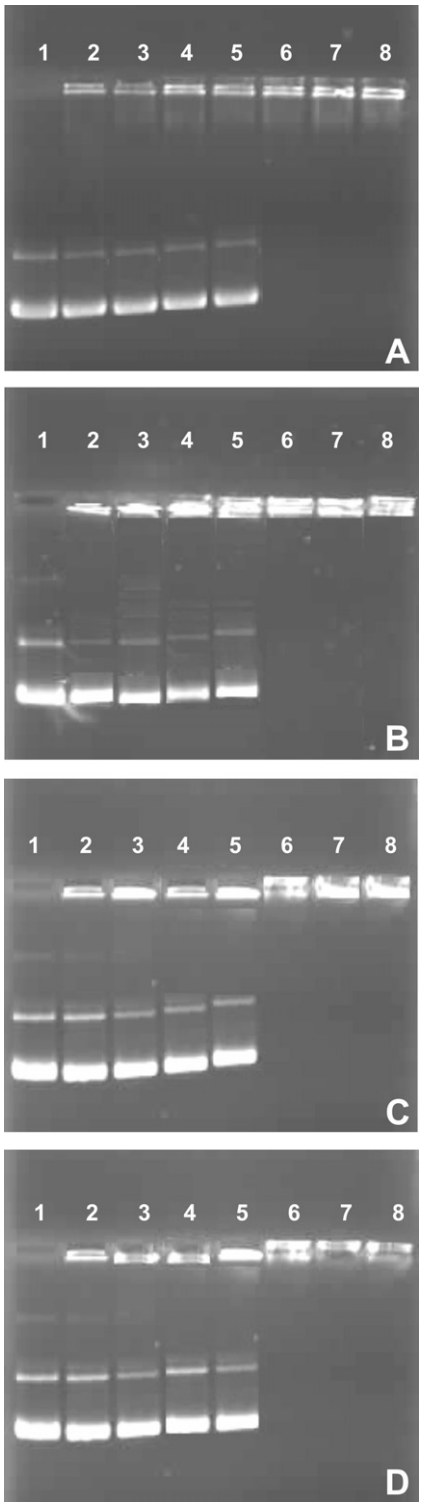


Fig. 3. Gel retardation study of (**A**) Cholαgal, (**B**) Cholβgal, (**C**) Cholαglu and (**D**) Cholβglu lipoplexes. Incubation mixtures (10 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposomes in lanes 1–8. (0, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µg), while the pGL3 DNA was kept constant throughout (0.5 µg).

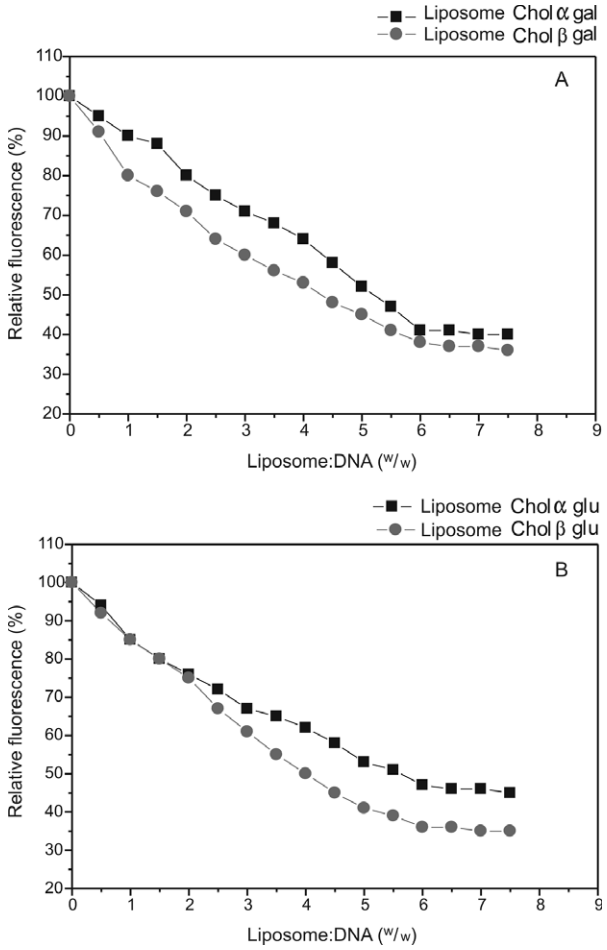


Fig. 4. Ethidium bromide intercalation/displacement assays. Incubation mixtures (500 µl) contained ethidium bromide (1 µg), pBR322 plasmid DNA (6 µg), HBS and increasing quantities of glycosylated cationic liposomes up to 45 µg. (**A**) Cholαgal and Cholβgal; (**B**) Cholαglu and Cholβglu.

negative charges forming ion pairs with the cationic head groups of MS09 (Figs 4A and 4B). Fluorescence intensity decreased steadily until a ratio was attained which correlated with total retardation seen for lipoplexes in gel retardation assays (6:1 w/w). Further addition of cationic liposome did not result in a reduction in fluorescence, indicating that no further compacting of the double-stranded nucleic acid had taken place. Cholβgal and Cholβglu liposomes both displayed higher displacement of the dye than their Cholαgal and Cholαglu counterparts, suggesting that β-anomers allow greater accessibility of cationic centres to the DNA than their corresponding α-anomers. On complexing with plasmid DNA, cationic liposomes yielded lipoplexes, which appeared as clusters or aggregates of globules, when viewed by TEM after negative staining, similar to those reported by others[39] and ourselves in unglycosylated MS09-containing lipoplexes.[25] At a liposome:DNA ratio of 5:1 (w/w), (+/–) charge ratio of 1.1, the diameter of all four lipoplexes ranged from 50 nm to 100 nm (see Fig. B(a), (c), (e) and (g) in online supplement), whereas at a higher liposome:DNA ratio of 6:1 (w/w), (+/–) charge ratio of 1.3, lipoplex diameters fell in the 80–200 nm range (Fig. B(b), (d), (f) and (h) online). In all complexes, small (10–20 nm) liposome-like vesicles were discernible.

Nuclease digestion assays

These studies enabled us further to characterize the strong binding interaction between the liposomes and the DNA and to observe what protection is afforded by the cationic liposomes to the bound DNA. The effect of the serum nucleases on two of the four lipoplexes can be seen in Figs 5A and B (Cholβgal and Cholβglu, respectively). Generally good protection was afforded to the DNA by the liposomes at liposome:DNA ratios of 3:1 to 7:1 (w/w). However, the greatest degradation of the DNA was noted for lipoplexes at a liposome:DNA ratio of 1:1 (w/w). This may be attributed to the low liposome:DNA (+/–) charge ratio at which there may be unbound DNA molecules present. Indeed, at such a low ratio it is anticipated that liposome-bound DNA may also be susceptible to serum nuclease digestion as

condensation may be incomplete. At higher charge ratios it can be seen that the DNA was well protected within the lipoplexes. The adsorption of serum proteins can induce a number of effects such as complex destabilization, aggregation or retargeting. The stability of lipoplexes or polyplexes depends on the strength of the electrostatic interaction, the total charge and the charge density of the carrier molecule.[40] In liver tissue a large proportion of acid DNAses is located in the sinusoidal cells,[41] a significant factor to be considered in the design of hepatotropic assemblies. Nuclease protection patterns in Cholαgal and Cholαglu lipoplexes were similar to those presented in Figs 5A and B (not shown).

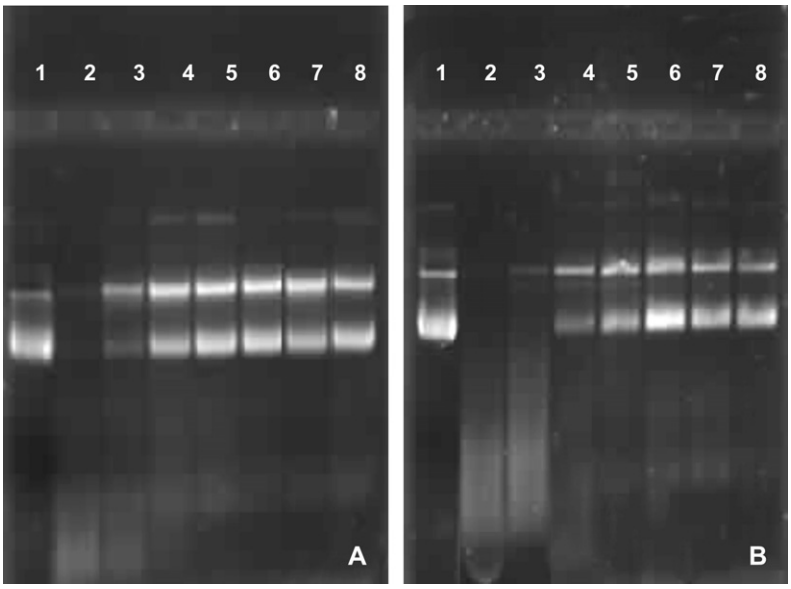


Fig. 5. Serum nuclease protection assays. Free or liposome-associated pGL3 DNA (1 μg) was exposed to 10% FBS in HBS for 4 hours at 37°C. Samples were then treated with SDS and EDTA prior to analysis by 1% agarose gel electrophoresis as described in Materials and methods. Lane 1, undigested plasmid DNA; Lanes 2–8, plasmid DNA with varying amounts of glycosylated cationic liposomes (0, 1.0, 2.0, 4.0, 5.0, 6.0, 7.0 μg). (**A**) Cholβgal; (**B**) Cholαglu.

Effect of lipoplexes on cell proliferation

To minimize possible perturbations arising from osmotic effects in toxicity assays and transfection experiments, lipoplexes were assembled in HEPES buffered saline, an environment in which the osmolality falls within the range found in MEM containing sodium bicarbonate or in mammalian blood. HepG2 and HeLa cells in 24-well plates were exposed to the same lipoplex concentration range chosen for *in vitro* transfection studies and for the same time. Growth inhibition effects in the two cell lines were very similar. At the highest liposome:DNA ratio of 7:1 (w/w) the four lipoplex series achieved a growth inhibition in the range 16–18% whilst at the optimal transfection ratio (5:1 w/w) growth inhibition ranged between 7 and 9% except in the case of the Cholαglu lipoplex (14%) (Fig. C(A) and (B) in online supplement). Results suggest that the lipoplexes are well tolerated by both cell lines.

Transfection studies

Galactosylated PEGylated polyplexes transfect hepatoma cells with a tenfold higher efficiency than corresponding glucosylated particles but show no difference in cell lines that lack the ASG-R.[42] Interestingly, it has been reported that replacement of galactose by glucose in PEI/DNA complexes (5% galactose) abolished transfection in HepG2 cells.[43] This trend was followed in the present study of the four glycosylated liposome formulations. Thus there was a fourfold increase in targeted transgene activity in HepG2 cells by the Cholβgal lipoplex (Fig. 6B) over its α-anomeric counterpart Cholαgal (Fig. 6A) at a liposome:DNA ratio of 5:1 (w/w), corresponding to a (+/–) charge ratio of 1.1. Furthermore, there was a greater than eightfold increase in activity of the Cholβgal lipoplexes over both Cholβglu and Cholαglu lipoplexes at the same charge ratios (Fig. 6C and D, respectively). The selectivity demonstrated by the ASG-R with respect to the glycopyranosyl moieties in glycosidic link with the membrane suggests that a spacer is not required for the hexoses to be accommodated in the binding site of the receptor. All four lipoplexes performed optimally at a liposome:DNA ratio of 5:1 (w/w), slightly below the ratio at which complete retention of DNA by liposomes was seen on gel retardation assays (6:1, w/w ; Fig. 3A–D). This may possibly be ascribed to the fact that the lipoplex size range at the liposome: DNA ratio of 5:1 (w/w) was somewhat smaller (50–100 nm) than that at the higher ratio (80–200 nm). It has recently been shown that galactosylated lipoplexes with a mean size of 141 nm lead to preferential transgene expression in parenchymal cells *in vivo*, while larger particles (235 nm) achieved higher transgene expression in non-parenchymal cells.[44] In addition, large galactosylated lipoplexes (150 nm) achieve limited penetration of the sinusoidal fenestrae in liver tissue.[45] Size reduction of galactosylated PEI/DNA complexes has also been shown to improve asialoglycoprotein receptor-mediated transfection of hepatocytes. Thus toroidal structures in the 50–80 nm size range with zeta potentials close to neutrality entered hepatocytes selectively and efficiently.[17] To demonstrate receptor-mediated endocytosis of directed DNA complexes, a competition assay in the presence of an excess of a cognate ligand was performed in HepG2 cells. In the presence of asialofetuin, a greater than 90% decrease in transgene activity was observed with all four lipoplexes, supporting the notion that the main route of delivery of the plasmid DNA was by receptor-mediation. These observations were corroborated by results from transfection studies in the HeLa cell line, which does not express the galactose-specific membrane lectin.[40] Here transfection levels were about 10% of those achieved in HepG2 cells at equivalent liposome:DNA ratios (Fig. 6A–D). Lipoplex stability and efficacy in serum is an important factor in the design of systems with the potential for application in whole organisms. Thus transfections were also conducted in the presence of 10% bovine serum. Transgene activity levels at the optimal liposome:DNA ratio (5:1, w/w) were reduced by 34, 45, 50 and 56% for Cholβgal, Cholαgal, Cholαglu and Cholβglu, respectively (Fig. 6A–D). Notably, Cholβgal lipoplexes, which promoted the highest levels of transgene activity, were also least affected by serum.

Conclusion

Stable galactosylated and glucosylated cationic liposomes have been prepared from the α- and β-anomers of the cholesteryl-D-glycopyranosides, the cationic cytofectin MS09 and the neutral co-lipid DOPE in a 1:4:4 mole ratio. The asialoorosomucoid receptor on the hepatocyte-like cell line HepG2 showed a clear anomeric preference for lipoplexes incorporating the β-anomer of cholesteryl-D-galactopyranoside (Cholβgal) as measured by the level of transgene activity. Moreover, the hydroxyl group orientation at the C-4 position of the pyranosides strongly influenced levels of transfection. The marked epimeric preference of the lectin for the galacto-

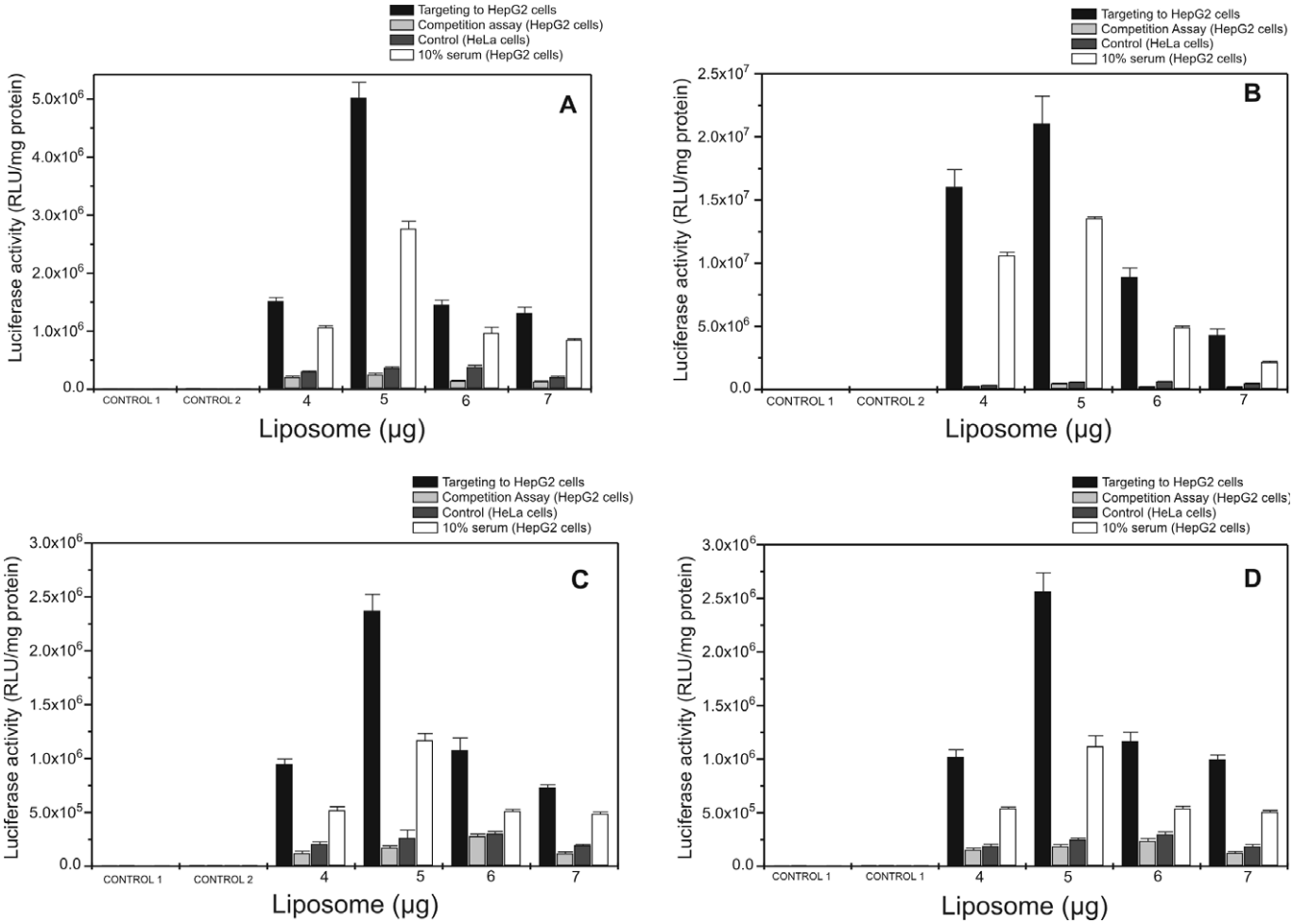


Fig. 6. Transfection studies of glycosylated cationic liposome:DNA lipoplexes in HepG2 and HeLa cell lines. Experiments were conducted with semi-confluent cells in 24-well plates. Cells were treated with lipoplexes comprising 1 µg pGL3 DNA and varying amounts of liposomes (0.0, 4.0, 5.0, 6.0, 7.0 µg) in 0.5 ml minimum essential medium for four hours. Thereafter, medium was removed and replaced with complete medium. Competition assays in the presence of 300 µg asialofetuin were conducted in the HepG2 cell line. In addition, transfections were conducted in HeLa cells, which are ASG-R negative. The effect of 10% FBS on transfection levels in HepG2 cells was also examined. Control 1 contained only HepG2 cells, while Control 2 contained HepG2 cells and naked pGL3 DNA. (**A**) Cholαgal; (**B**) Cholβgal; (**C**) Cholαglu; (**D**) Cholβglu. Data are presented as means ± s.d. (n = 4).

pyranosides over the glucopyranosides was evident even in the absence of a spacer between the carbohydrate moieties and the membrane-anchored cholesteryl component. These results comprehensively indicate that the Cholβgal lipoplexes are suitable candidates for further development.

Supplementary material is available in the online version of this paper at www.sajs.co.za

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Supplementary material to:

Singh M., Rogers C.B. and Ariatti M. (2007). Targeting of glycosylated lipoplexes in HepG2 cells: Anomeric and C-4 epimeric preference of the asialoglycoprotein receptor. *S. Afr. J. Sci.* **103**, 204–210.

Spectral data

Cholesteryl-β-D-tetra-O-acetylgalactopyranoside. M.p. 157–159°C; ^{1}H NMR (300MHz, $CDCl_3$): δ 0.65 (s,3H,C-CH_3), 0.83 (d, 6H, J = 6.6 Hz, CH-CH_3), 0.89 (d, 3H, J = 6.5, CH-CH_3), 0.96 (s, 3H, C-CH_3), 1.96-2.12 (12H, 4 × CO-CH_3), 3.47 (m, 1H, Chol-$H_{3\alpha}$), 3.86 (t, J = 7.0, H-5′), 4.05-4.19 (m, 2H, H-6′a, H-6′b), 4.52 (d, 1H, J = 8.0 Jz, H-1′), 4.99 (dd, 1H, J = 10.4, 3.4 Jz, H-3′), 5.16 (dd, 1H, J = 10.4, 7.9 Jz, H-2′), 5.35 (m, 2H, H-4, Chol-H_6). ^{13}C NMR (75 MHz, $CDCl_3$): sugar region: δ 100.29 (C-1′), 69.07 (C-2′), 70.55 (C-3′), 67.03 (C-4′), 71.02 (C-5′), 61.30 (C-6′). Cholesteryl moiety: δ 11.86 (C-18), 18.72 (C-21), 19.36 (C-19), 21.05 (C-11), 22.57 (C-26), 22.83 (C-27), 24.29 (C-15), 28.23 (C-16), 29.52 (C-2), 23.82 (C-23), 28.02 (C-25), 31.86 (C-8), 31.94 (C-7), 35.78 (C-20), 36.18 (C-22), 36.71 (C-10), 39.52 (C-24), 39.74 (C-12), 37.19 (C-1), 38.96 (C-4), 42.32 (C-13), 50.14 (C-9), 56.74 (C-14), 56.14 (C-17), 80.35 (C-3), 122.19 (C-6), 140.33 (C-5).

Cholesteryl-α-D-tetra-O-acetylgalactopyranoside. M.p. 194–197°C; ^{1}H NMR (300MHz, $CDCl_3$): δ 0.65 (s,3H,C-CH_3), 0.84 (d, 6H, J = 6.6 Jz, CH-CH_3), 0.89 (d, 3H, J = 6.5, CH-CH_3), 0.98 (s, 3H, C-CH_3), 1.97-2.12 (12H, 4 × CO-CH_3), 3.43 (m, 1H, Chol-$H_{3\alpha}$), 4.07 (t, 2H, J = 7.0, 5.4, H-6′a, H-6′b), 4.32 (m, 1H, H-5′), 5.43 (m, 1H, H-1′).

Cholesteryl-β-D-tetra-O-acetylglucopyranoside. M.p. 160–163°C (Seo *et al.*[27] reported 160–164°C); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.65 (s,3H,C-CH_3), 0.83 (d, 6H, J = 6.6 Jz, CH-CH_3), 0.88 (d, 3H, J = 6.5, CH-CH_3), 0.96 (s, 3H, C-CH_3), 2.00-2.05 (12H, 4 × CO-CH_3), 3.45 (m, 1H, Chol-$H_{3\alpha}$), 4.08 (m, 2H, H-6′a, H-6′b), 3.65 (dd, 1H, J = 7.5,2.3, H-5′), 4.93 (dd,1H, J = 9.5,8.0, H-3′), 4.57 (d, J = 7.9 Jz, H-1′), 5.18 (t, 1H, J = 9.4, H-2′), 5.33 (d, 1H, J = 5.0, Chol-H_6), 5.05 (t, 1H, J = 9.6, H-4′).

^{13}C NMR (75 MHz, $CDCl_3$): sugar region: δ 99.64 (C-1′), 71.68 (C-2′), 72.90 (C-3′), 68.50 (C-4′), 71.47 (C-5′), 62.09 (C-6′). Cholesteryl moiety: δ 11.85 (C-18), 18.72 (C-21), 19.36 (C-19), 21.04 (C-11), 22.57 (C-26), 22.83 (C-27), 24.28 (C-15), 28.23 (C-16), 29.44 (C-2), 23.81 (C-23), 28.02 (C-25), 31.85 (C-8), 31.94 (C-7), 35.78 (C-20), 36.18 (C-22), 36.71 (C-10), 39.52 (C-24), 39.74 (C-12), 37.19 (C-1), 38.91 (C-4), 42.32 (C-13), 50.14 (C-9), 56.74 (C-14), 56.14 (C-17), 80.09 (C-3), 122.18 (C-6), 140.34 (C-5).

Cholesteryl-α-D-tetra-O-acetylglucopyranoside. M.p. 201–203°C (Seo *et al.*[27] reported 202–204°C) ^{1}H NMR (300MHz, $CDCl_3$): δ 0.65 (s,3H,C-CH_3), 0.84 (d, 6H, J = 6.5 Jz, CH-CH_3), 0.89 (d, 3H, J = 6.5, CH-CH_3), 0.96 (s, 3H, C-CH_3), 1.99-2.06 (12H, 4 × CO-CH_3), 3.43 (m, 1H, Chol-$H_{3\alpha}$), 4.06-4.21 (m, 2H, H-6′a, H-6′b), 5.32 (d, 1H, Chol-H_6), 5.46 (t, 1H, H-1′). ^{13}C NMR (75 MHz, $CDCl_3$): sugar region: δ 94.23 (C-1′), 71.04 (C-2′), 70.22 (C-3′), 68.74 (C-4′), 67.21 (C-5′), 67.07 (C-6′). Cholesteryl moiety: δ 11.86 (C-18), 18.72 (C-21), 19.38 (C-19), 21.04 (C-11), 22.57 (C-26), 22.84 (C-27), 23.82(C-23), 24.29 (C-15), 28.02 (C-25), 28.24 (C-16), 31.86 (C-8), 37.20(C-1), 39.52 (C-24), 39.73 (C-12), 42.31 (C-13), 50.09 (C-9), 56.13 (C-17), 56.70 (C-14), 122.21 (C-6), 140.30 (C-5).

Cholesteryl-β-D-galactopyranoside (**Cholβgal**). M.p. 270–273°C. ^{1}H NMR (300 MHz, $CDCl_3$, C_5D_5N): δ 0.58 (s,3H,C-CH_3), 0.84 (d, 6H, J = 4.0 Jz, CH-CH_3), 0.88 (d, 3H, J = 6.5, CH-CH_3), 4.35 (d, 1H, H-1′). ^{13}C NMR (75 MHz, $CDCl_3$, C_5D_5N): sugar region: δ 102.26 (C-1′), 74.84 (C-2′), 78.63 (C-3′), 71.43 (C-4′), 78.09 (C-5′), 62.61 (C-6′). Cholesteryl moiety: δ 11.88 (C-18), 18.80 (C-21), 19.31 (C-19), 21.11 (C-11), 22.60 (C-26), 22.86 (C-27), 23.95 (C-23), 24.35 (C-15), 28.08 (C-25), 28.35 (C-16), 31.89 (C-8), 37.30 (C-1), 39.57 (C-24), 39.81 (C-12), 42.33 (C-13), 50.18 (C-9), 56.18 (C-17), 56.69(C-14), 121.75 (C-6), 140.65 (C-5).

Cholesteryl-α-D-galactopyranoside (**Cholβgal**). ^{1}H NMR (300 MHz, $CDCl_3$, C_5D_5N): δ 0.58 (s,3H,C-CH_3), 0.81(d, 6H, J = 5.6, CH-CH_3), 0.85 (d, 3H, J = 7.0, CH-CH_3), 0.89 (s, 3H, C-CH_3), 5.29 (d, 1H, J = 3.8, H-1′). ^{13}C NMR (75 MHz, $CDCl_3$, C_5D_5N): sugar region: δ 98.29 (C-1′), 76.51 (C-5′), 75.03 (C-3′), 72.32 (C-2′), 69.86 (C-4′), 62.03 (C-6′). Cholesteryl moiety: δ 11.87 (C-18), 18.81 (C-21), 19.31 (C-19), 21.11 (C-11), 22.60 (C-26), 22.85 (C-27), 23.96 (C-23), 24.35 (C-15), 28.07 (C-25), 28.35 (C-16), 31.91 (C-8), 37.16 (C-1), 39.57 (C-24), 39.81 (C-12), 42.33 (C-13), 50.13 (C-9), 56.18 (C-17), 56.70 (C-14), 121.63 (C-6), 140.89 (C-5).

Cholesteryl-β-D-glucopyranoside (**Cholβglu**). M.p. 256–260°C (Seo *et al.*[27] reported 257–260°C). ^{1}H NMR (300 MHz, C_5D_5N): δ 0.54 (s,3H,C-CH_3), 0.77 (d, 6H, J = 6.6 Jz, CH-CH_3), 0.85 (d, 3H, J = 6.5, CH-CH_3), 4.85 (d, 1H, J = 7.6, H-1′). ^{13}C NMR (75 MHz, C_5D_5N): sugar region: δ 102.84 (C-1′), 76.51 (C-5′), 75.03 (C-3′), 72.32 (C-2′), 69.86 (C-4′), 62.03 (C-6′). Cholesteryl moiety: δ 11.76 (C-18), 18.72 (C-21), 19.20 (C-19), 21.05 (C-11), 22.47 (C-26), 22.72 (C-27), 23.87 (C-23), 24.26 (C-15), 27.98 (C-25), 28.29 (C-16), 31.82 (C-8), 37.26 (C-1), 39.48 (C-24), 39.73 (C-12), 42.24 (C-13), 50.12 (C-9), 56.10 (C-17), 56.60 (C-14), 121.66 (C-6), 140.77 (C-5).

Cholesteryl-α-D-glucopyranoside (**Cholβglu**). ^{13}C NMR (75 MHz, C_5D_5N): sugar region: δ 98.81 (C-1′), 73.18 (C-5′), 72.50 (C-3′), 71.39 (C-4′), 70.62 (C-2′), 62.21 (C-6′). Cholesteryl moiety: δ 11.76 (C-18), 18.72 (C-21), 19.24 (C-19), 21.04 (C-11), 22.47 (C-26), 22.72 (C-27), 23.87 (C-23), 24.27 (C-15), 27.98 (C-25), 28.28 (C-16), 31.84 (C-8), 37.14 (C-1), 39.47 (C-24), 39.73 (C-12), 42.24 (C-13), 50.08 (C-9), 56.10 (C-17), 56.60 (C-14), 121.61 (C-6), 140.98 (C-5).

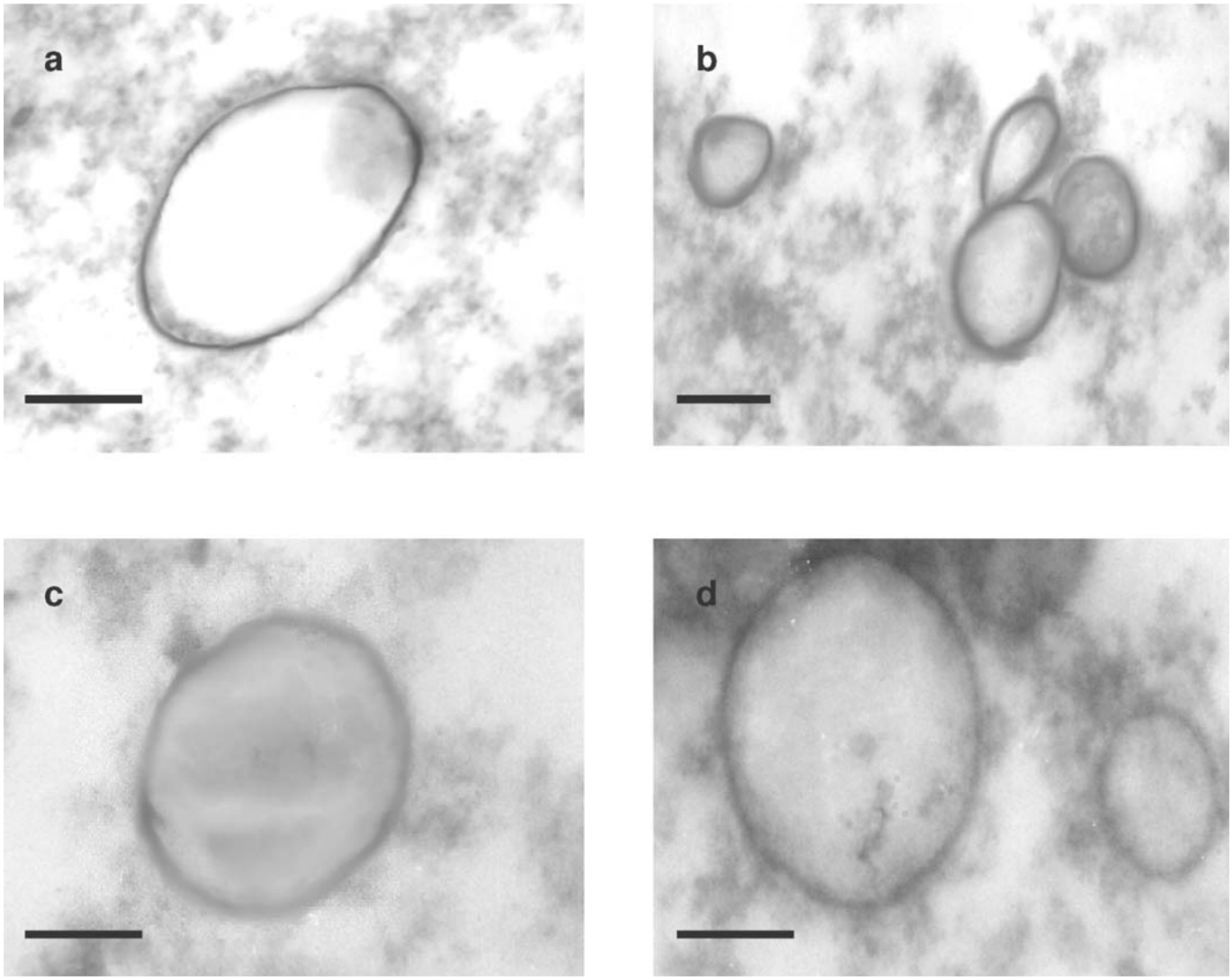

Fig. A. Transmission electron micrographs of unilamellar liposomes prepared from MS09, DOPE and cholesteryl glycosides in 4:4:1 molar ratio. (**a**) Cholαgal; (**b**) Cholβgal; (**c**) Cholαglu; (**d**) Cholβglu. Bar = 100 nm.

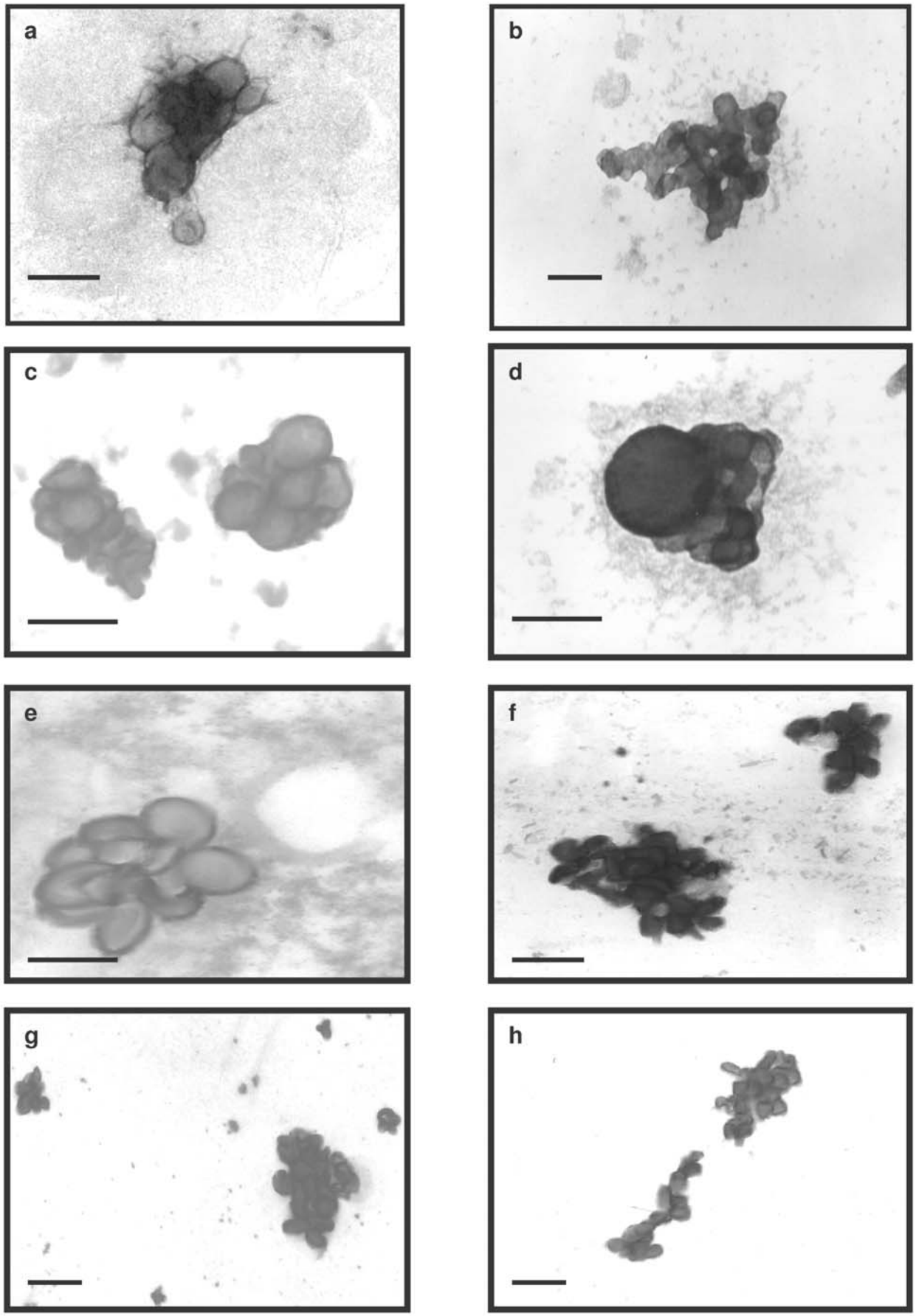

Fig. B. Transmission electron micrographs of lipoplexes formed between glycosylated cationic liposomes and pGL3 DNA. (**a**) and (**b**) Cholαgal; (**c**) and (**d**) Cholβgal; (**e**) and (**f**) Cholαglu; (**g**) and (**h**) Cholβglu. Liposome:DNA ratios were set at 5:1 (w/w) (a), (c), (e) and (g) or 6:1 (w/w) (b), (d), (f) and (h). Bar = 50 nm.

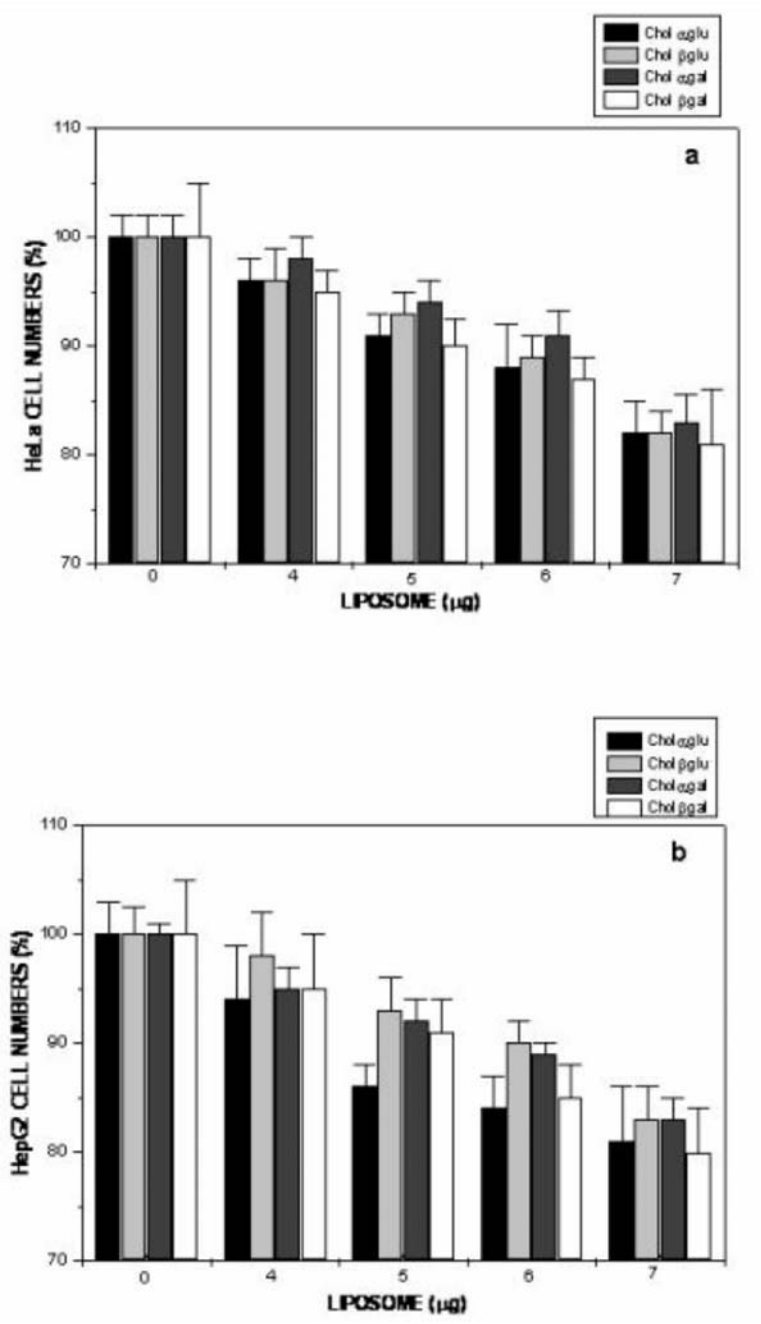

Fig. C. Growth inhibition assays. Semiconfluent cells in 24-well plates were treated with lipoplexes in which glycosylated cationic liposomes were varied in quantity (4.0, 5.0, 6.0, 7.0 μg/well) while pGL3 was kept constant (1 μg/well) in a total volume of 0.5 ml of minimum essential medium. Incubation for the first four hours was in the absence of FBS. Thereafter, the medium was replaced by complete medium. Control wells (no liposomes or DNA) were assumed to show 100% survival. (**a**) HepG2 cells; (**b**) HeLa cells. Data are presented as means ± s.d. ($n = 4$).