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Full Length Research Paper

The effect of pegylation on the transfection activity of two homologous cationic cholesteryl cytofectins

Moganavelli Singh, Justine Borain, Naeema Noor-Mahomed and Mario Ariatti*

Non-Viral Gene Delivery Laboratory, Discipline of Biochemistry, Westville Campus, University of KwaZulu-Natal, Durban, South Africa.

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Polyethylene glycol is being used increasingly to improve circulation times and enhance bioavailability of therapeutic molecules and nano sized macromolecular assemblies. Here, two homologous cationic cholesteryl cytofectins 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) with 2 and 3 carbon spacer elements, respectively, have been formulated into liposomes with near equimolar amounts of dioleoylphosphatidyl ethanolamine (DOPE) and 2 and 5% polyethylene glycol$_{2000}$ (PEG$_{2000}$). Pegylated liposomes (80 - 150 nm diameter) formed electrostatic complexes with plasmid DNA in the 1.5:1 – 2.5:1 range of liposome (positive): DNA (negative) charge ratio, which afforded protection to the DNA cargo against serum nuclease digestion. Plasmid pGL3-containing pegylated lipoplexes were only weakly cytotoxic in the human embryonic kidney cell line HEK 293. Gene transfer experiments in this cell line confirmed that the homologue with the 3 carbon spacer Chol-T, is associated with higher transgene activity, a trend which has been previously observed in unpegylated lipoplexes. Furthermore, only a 15% drop in transfection activity was recorded in increase of pegylation level from 2 to 5 mole percent.

Key words: Cationic cytofectin, gene transfer, polyethylene glycol, HEK 293.

INTRODUCTION

The nucleic acid therapeutic approach for the treatment of major diseases including cancer is poised to revolutionize medicine. Thus, gene therapy or the introduction of corrective DNA into diseased cells may be effected by two main approaches. Viral vectors, which have been extensively investigated, generally promote high gene transfer efficiency but are hampered by safety concerns relating to mutagenesis, carcinogenesis and immunostimulation (Stein and Walther, 2000; Charudharshini and Burgess, 2009). Amongst the more common non-viral vectors, are the cationic lipids which exhibit low toxicity and low immunoresponse (Woodle, 1995: Li et al., 1996; Maitani et al., 2007). Cationic cholesteryl cyto-fectins (CCCs) form an important class of amphiphilic lipid vectors that show great potential. Molecules of this description (Figure 1) are formulated with a neutral co-lipid, usually dioleoylphosphatidyl ethanolamine (DOPE), into cationic liposomes which form non-covalent electrostatic complexes with polyanionic DNA or RNA (Gao and Huang, 1991). These lipoplexes are capable of entry into mammalian cells predominantly by endocytosis (Mashkevich, 2007). This has facilitated the development of such systems for *in vivo* gene delivery. In the quest to produce cytofectins which afford liposomes with high transfection potential, the nature of the linkage, spacer length and cationic head group have been examined (Figure 1).

Thus, it has been shown that the ether linkage between the hydrophobic cholesteryl skeleton and the spacer affords compounds which lead to higher transfection

*Corresponding author. E-mail: ariattim@ukzn.ac.za. Tel: +27 (0)31 2607981. Fax: +27 (0)31 2607942.

Abbreviations: CCCs, Cationic cholesteryl cytofectins; **DC-Chol,** 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol; **Chol-T,** 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol; **DOPE,** dioleoylphosphatidyl ethanolamine; **PEG,** polyethylene glycol; **PEG$_{2000}$DSPE,** polyethylene glycol$_{2000}$-distearoylphosphatidyl ethanolamine; **HEK 293,** human embryonic kidney cell line; **HEPES,** ethidium bromide; 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid; **MTT,** 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; **PBS,** phosphate buffered saline; **FBS,** foetal bovine serum; **BCA,** bicinchoninic acid; **MEM,** minimum essential medium.

Table 1. Liposome and lipoplex compositions.

Liposome preparation	Liposome components (µmoles)				Optimal liposome : DNA binding ratio (µg/µg)	Liposome : DNA charge ratio (+ve:-ve)
	Chol-T	DC-Chol	DOPE	PEG_{2000}DSPE		
1A	2	-	1.92	0.08	7:1	1.8:1
1B	2	-	1.80	0.20	10:1	2.5:1
2A	-	2	1.92	0.08	6:1	1.5:1
2B	-	2	1.80	0.20	7:1	1.9:1

levels than similar derivatives based on ester and urethane connectors (Ghosh et al., 2000).

The head group varies considerably amongst CCCs. While primary amino functionality leads to effective liposomes (Singh and Chaudhuri, 2004; Singh and Ariatti, 2006; Kim et al., 2009), tertiary amino and quaternary ammonium functionalities are favoured (Gao and Huang, 1991; Okayama et al., 1997; Singh et al., 2001; Cao et al., 2000; Lesage et al., 2002; Reynier et al., 2004; Bajaj et al., 2008). There is strong evidence, which suggests that the most active quaternary ammonium head group is the dimethyl hydroxyethyl alkyl ammonium function (Percot et al., 2004; Ding et al., 2008). A large number of CCCs feature 2C (ethano) and 3C (propane) spacer elements (Gao and Huang, 1991; Okayama et al., 1997; Hasegawa et al., 2002; Nakanishi, 2003). Studies with CCCs bearing dimethylamino head groups show that the 3C spacer promotes higher transfection activity in NIH 3T3 and HeLa cells *in vitro* (Takeuchi et al., 1996). A similar trend has been observed in derivatives displaying quaternary ammonium head groups in the human melanoma cell line B16-F10 (Reynier et al., 2004).

The development of CCC-based non-viral systems for *in vivo* gene delivery is confronted and mitigated by the undesirable interaction of lipoplexes with serum proteins and by aggregation (Anchordoquy and Xu, 2008). The incorporation of non-toxic, biocompatible polymers into lipoplexes has been advanced to obviate such events. Notably, polyethylene glycol (PEG) has been anchored to liposome membranes to afford a hydrophilic protective cloud to lipoplexes, which confers steric repulsion to vesicles and promotes prolonged liposome circulation in the blood (Byun et al., 2009).

In this study, we reported the effect that 2 and 5 mole percentage polyethylene $glycol_{2000}$ (PEG_{2000}) has on the CCC spacer length preference in the transfection of the human embryonic kidney cell line HEK 293 with plasmid pGL3 lipoplexes. We also discuss the effect that the degree of liposome pegylation has on the stoichiometry of liposome: plasmid DNA complexes.

MATERIALS AND METHODS

Materials

Polyethylene $glycol_{2000}$-distearoylphosphatidyl ethanolamine (PEG_{2000}DSPE) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Dioleoylphosphatidyl ethanolamine (DOPE) and bicinchoninic acid (BCA) were purchased from Sigma-Aldrich (Steinheim, Germany). Ethidium bromide, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid (HEPES), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and phosphate buffered saline (PBS) were from Merck (Darmstadt, Germany). Plasmid pGL3 was purchased from Promega (Madison, WI, USA). Plasmid pBR322 was from Roche Applied Science (Gauteng, South Africa). Minimum essential medium (MEM) and heat inactivated foetal bovine serum (FBS) were obtained from Gibco (Invitrogen Life Sciences). 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) were prepared by the method of Gao and Huang (1991) from cholesteryl chloroformate and the respective dimethylaminoalkylamine.

Preparation of pegylated cationic liposomes

Mixtures of DC-Chol and Chol-T with DOPE and PEG_{2000}-DSPE in ratios given in Table 1 were dissolved in dry chloroform (1 ml). The components were deposited as a thin film in a test tube by rotary evaporation of the solvent *in vacuo* at 21°C. The film was subjected to high vacuum for 2 h to remove all traces of solvent. Thereafter, the film was hydrated in sterile 20 mM HEPES, 150 mM NaCl (HBS, pH 7.5, 1 ml) at 4°C for 24 h and briefly vortexed. Finally, the suspension was sonicated in an ELMA Transsonic bath type sonicator (T 460/H) for 5 min to afford unilamellar liposomes. Liposomes were routinely stored at 4°C and remained stable for several months (routine DNA binding analyses).

Transmission electron microscopy

Liposome suspensions (2.5 µg/µl, 50 µl) were placed on carbon-coated copper grids. After 30 s, grids were stained with phosphotungstic acid solution (2% w/v, 50 µl). Excess liquid was removed after 1 min and grids were air dried at room temperature before viewing in a Jeol 1010 Megaview Soft Imaging Transmission Electron Microscope system operating at 80 kV.

Band shift assays

Plasmid pBR322 DNA (0.5 µg) was incubated with increasing amounts of the pegylated liposome preparations up to 5 µg in HBS (8 µl) at 21°C for 20 min. Gel loading buffer was then added (0.05% bromophenol blue, 40% sucrose, 2 µl) and samples were subjected to electrophoresis (40 V) on 1% agarose gels containing ethidium bromide (1.5 µg/ml) in a buffer containing 36 mM Tris-HCl, 30 mM sodium phosphate and 10 mM EDTA (pH 7.5) for 90 min. Gels were viewed in a Syngene G-box under transillumination at 300 nm.

Nuclease digestion assays on lipoplexes

Mixtures of pGL3 plasmid (1 µg) and pegylated liposomes (4 - 11

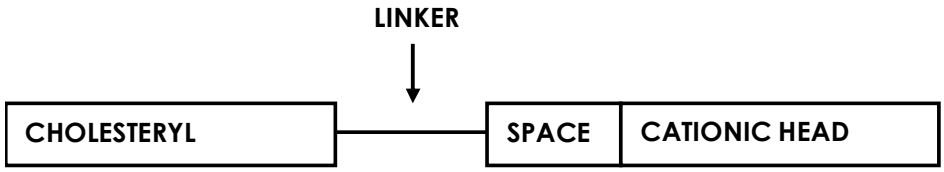

Figure 1. A schematic representation of the structural elements of a cationic cholesteryl cytofectin.

µg) were prepared in HBS (10 µl, pH7.5). After 30 min at room temperature, FBS was added to a final concentration of 10% v/v. Mixtures were incubated at 37°C for 4 h before addition of EDTA to a concentration of 10 mM and sodium dodecyl sulphate to 0.5% w/v. Suspensions were incubated further at 55°C for 20 min and thereafter, subjected to electrophoresis on 1% agarose gels as described for the band shift assays.

Cell culture

HEK 293 cells (University of the Witwatersrand, Johannesburg, South Africa) were propagated at 37°C in 25 cm^2 screw cap flasks in 5 ml MEM with Earle's salts supplemented with penicillin G (100 U/ml), streptomycin (100 µg/ml), 20 mM HEPES (pH 7.5) and FBS (10%). Cultures were routinely trypsinized in 0.25% (w/v) trypsin, 0.1% EDTA (Whittaker, M.A. Bioproducts, MD, USA) and passaged 1:4 every 4 days.

Amplification of pGL3 DNA

Plasmid pGL3 was amplified in *Escherichia coli* strain JM109 and isolated from cultures using a Nucleobond® AX PC 100 kit (Germany). Preparations regularly gave 260/280 nm ratios > 1.8 and were diluted with 18 Mohm water to a concentration of 0.5 µg/µl. Stocks were stored in 500 µl aliquots at -80°C.

Cytotoxicity assays

The cytotoxicity of lipoplexes towards HEK 293 cells was determined under transfection conditions. Cells (2.0 x 10^3/well) were seeded into 48 well plates and incubated in complete medium for 24 h. Lipoplexes were then prepared at various liposome : DNA ratios about the optimal DNA binding ratio for each liposome preparation. Cells were then prepared by replacing complete medium with MEM (200 µl/well). Lipoplexes were added to wells and plates were incubated for 4 h at 37°C. Thereafter, medium was removed and replaced with complete medium (200 µl/well). After incubation for a further 36 h, medium was removed and MTT solution (5 mg/ml in phosphate buffered saline, 200 µl) was added. Cells were incubated for 4 h to allow for the formation of blue formazan crystals. The MTT solution was then replaced with DMSO (200 µl/well) to dissolve the formazan crystals. Absorbance of extracts was then measured at 570 nm. The percentage cell viability was then calculated as follows:

$$\text{Percentage cell viability} = [A_{570} \text{ treated cells} - \text{Background}]/[A_{570} \text{ control cells} - \text{Background}] \times 100$$

Transfection of HEK 293 cells

Transfection complexes containing pGL3 plasmid (0.5 µg) and varying amounts of 2 and 5% pegylated liposomes (4 - 11 µg) in HBS (10 µl, pH 7.5) were incubated for 30 min immediately prior to cell exposure. Cells (2.2 x 10^3/well) were seeded into 48 well plates and incubated as described above. Wells were then drained of medium and serum-free medium (200 µl) was added. Following the addition of lipoplexes, cells were incubated for 4 h at 37°C. Medium was removed and complete medium was added. After 36 h at 37°C, the medium was again removed and cells were assayed for transgene activity.

Measurement of luciferase activity in transfected cells

Cells which had been exposed to transfecting lipoplexes were lysed with Promega lysis buffer (5 mM Tris-phosphate, 0.4 mM dithiothreitol, 0.4 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 2.5% glycerol and 0.2% Triton X-100, 80 µl). Plates were placed on a platform rocker for 20 min at 30 revolutions/minute. Lysates were spun at 12 000xg for 30 s to obtain clear extracts. Luciferase activity in 20 µl extracts was determined by the Promega Luciferase Assay system according to the manufacturer's instructions. Luminescence was measured on a Lumac Biocounter 1500 as relative light units (RLU). Protein concentration in extracts was determined by the BCA assay (Sigma) with BSA as standard protein.

RESULTS AND DISCUSSION

DC-Chol (Gao and Huang, 1991) is a CCC featuring a hydrophobic cholesteryl moiety, a biodegradable carbamoyl linker (Lee et al., 2005) a dimethylamino cationic head group and a 2C ethane spacer element (Figure 1). Its higher homologue Chol-T differs only in the spacer length, which is a 3C propane. These cholesteryl derivatives form stable unilamellar liposomes when formulated with equimolar amounts of the neutral co-lipid DOPE, which lends stability to the liposome and improves cytosolic delivery of liposome-bound DNA (Li et al., 1996). More particularly, DOPE facilitates the formation of lipid bilayers with the CCCs and destabilizes the endosomal membranes thus facilitating release of DNA from lipoplexes into the cytosol (Xu and Szoka, 1996). We report here on the effect of pegylation on the transfection activity of liposomes containing DOPE and DC-Chol or Chol-T in the human embryonic kidney cell line HEK 293. Hence, four liposome formulations were prepared from the two CCCs with PEG_{2000}DSPE present at 2 and 5 mole percentage (Table 1). Liposomes were established to be in the 80 - 150 nm size range by trans-

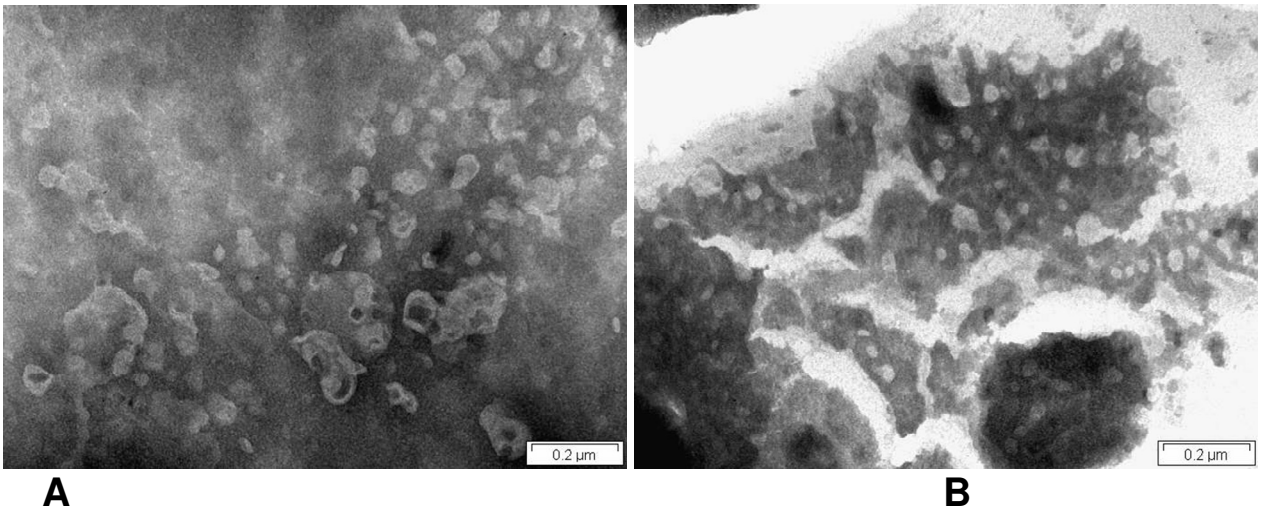

Figure 2. Representative transmission electron micrographs of pegylated liposomes. A) 2% pegylated DC-Chol liposomes; B) 5% pegylated DC-Chol liposomes. Bar = 200 nm.

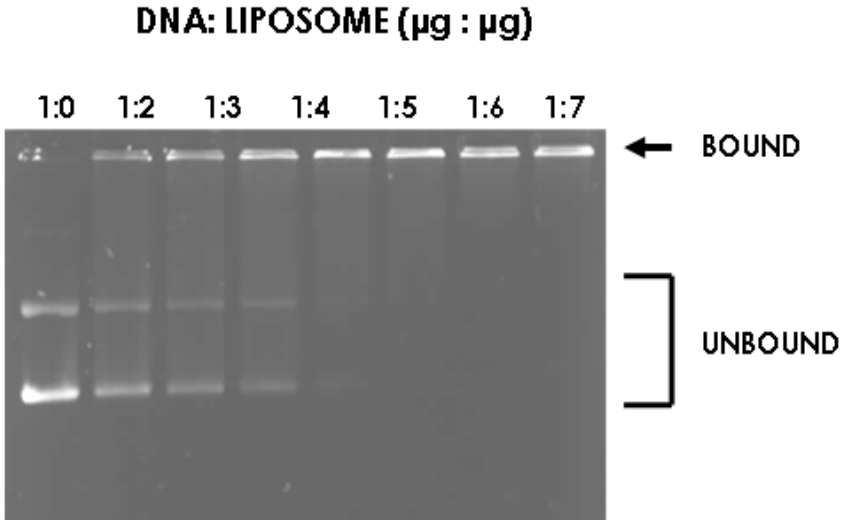

Figure 3. Representative band shift assay. Incubation mixtures (8 µl) contained pBR322 DNA (0.5 µg) and the following 2% pegylated DC-Chol liposome : DNA ratios (w/w): Lanes 1 - 8 (0:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1 and 8:1 respectively).

mission electron microscopy (Figure 2) and remained stably suspended when stored at 4°C for several months. The DNA binding capacity of liposomes was determined in band shift assays in which liposome-bound DNA fails to migrate into the agarose matrix, as complexes are too large or electroneutral and free DNA is seen as discrete bands (Figure 3). This permits the determination of the optimal DNA-binding ratio for each liposome preparation (Table 1). It is of interest to note that for both Chol-T and DC-Chol liposomes, the ratio is higher for the 5% pegylated preparations. This suggests that the cationic charges on the bilayer surface of the liposomes are relatively more adumbrated than in the 2% pegylated preparations. Therefore higher levels of 5% pegylated liposomes are required to bind the plasmid DNA. This is reflected also in the liposome (+ve) : DNA (-ve) charge ratios at full retardation of DNA in band shift assays. Hence Chol-T and DC-Chol 2% pegylated liposomes fully retarded at 1.8:1 and 1.5:1, while at 5% pegylation, the ratios are 2.5:1 and 1.9:1, respectively. The *in vitro* and *in vivo* application of lipoplexes for gene delivery requires that the DNA cargo remains intact in the extracellular environment although serum DNases are present. In Figure 4, we showed the protection offered to plasmid DNA by liposomes over a range of liposome : DNA ratios in the presence of 10% FBS for 4 h at 37°C. Under these conditions, free DNA is seen to be fully degraded (Figure 4, lane 2).

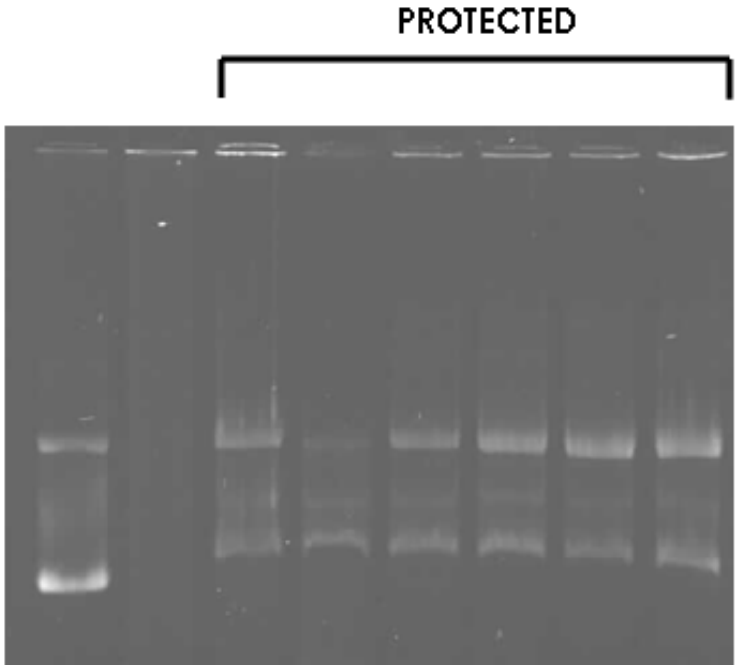

Figure 4. Nuclease protection assay. Plasmid pGL3 DNA (1 µg) was incubated in the presence of 2% pegylated DC-Chol liposomes: Lanes 3 - 5 (4, 5, 6 µg) and 5% pegylated DC-Chol pegylated liposomes: Lanes 6 - 8 (6, 7, 8 µg). Lane 1, marker plasmid DNA; lane 2, unprotected DNA.

The cytotoxicities of lipoplexes at varying liposome : DNA ratios were determined under transfection conditions using the MTT assay. Results presented in Figures 5A - D show that at the optimal liposome : DNA ratios, cell viability for all four lipoplex preparations lay in the range 88 - 91% of control untreated cells. The 5% pegylated DC-Chol lipoplexes at a liposome : DNA ratio of 8:1 proved to be the most cytotoxic complexes (77% viability). The findings indicate that 2 and 5% pegylated CCC lipoplexes are generally well tolerated in the HEK 293 cell line. Transfection levels were assessed by measuring luciferase activity following gene transfer experiments with plasmid pGL3. Highest transgene activity was attained by 2% pegylated Chol-T lipoplexes (1A) at the liposome : DNA ratio of 8:1. This was 2.5 times greater than levels achieved by the 2% pegylated DC-Chol lipoplexes (2A) and 3 times greater than 5% pegylated DC-Chol (2B) (Figure 6). Results presented here support the notion that higher pegylation levels lead to reduced transfection activity *in vitro* although it must be added that in this work we have observed only a moderate reduction in transfection activity on increase of pegylation level from 2 to 5% (approximately 15%). It has been suggested that the inhibitory effect of PEG on transfection activity does not reflect reduced uptake of lipoplexes but indicates a diminished interaction of the liposome lipids with the endosomal membrane. If DOPE is prevented from destabilizing the endosomal membrane, less DNA is released into the cytosol and a greater proportion is degraded upon vesicular fusion with the lysosome (Remaut et al., 2007). It has however, been suggested by others that liposome uptake may be sterically inhibited by PEG (Deshpande et al., 2004). Pegylation levels selected for this study were set at 2 and 5 mole percentage. This is within the range believed to afford the highest biological activity to liposomes (1.9 - 10%) (Peeters et al., 2007). Although PEG is non-toxic, non-immunogenic and easily excreted (Ishida and Kiwada, 2008; Zalipsky, 1995), the molecular size is an important factor in liposome formulations. Thus PEG_{2000} has been shown to extend 6.0 nm from the liposome bilayer and is favoured over PEG_{5000}, which extends 10.0 nm and therefore contributes more to the hydrodynamic radius of the liposome (Kenworthy et al., 1995). The polymer prolongs liposome circulation time *in vivo* (Allen et al., 1991), inhibits protein absorption on the surface of liposomes and recognition by the reticuloendothelial system (Gabizon, 2001; Garinot et al., 2007) and provides a steric barrier against opsonins (Ishida and Kiwada, 2008; Managit et al., 2003). PEG_{2000} was selected for this study as it reflects a compromise between an effective steric barrier and a long circulation half life (Allen, 1994; Song et al., 2002). Studies have shown that up to a 7 mole percentage of PEG_{2000}, the bilayer extra vesicular surface remains relatively exposed (Needham et al., 1997). This would permit interaction of DNA with CCC head groups which, in the case of DC-Chol and Chol-T, extend approximately 6 Å from the membrane-

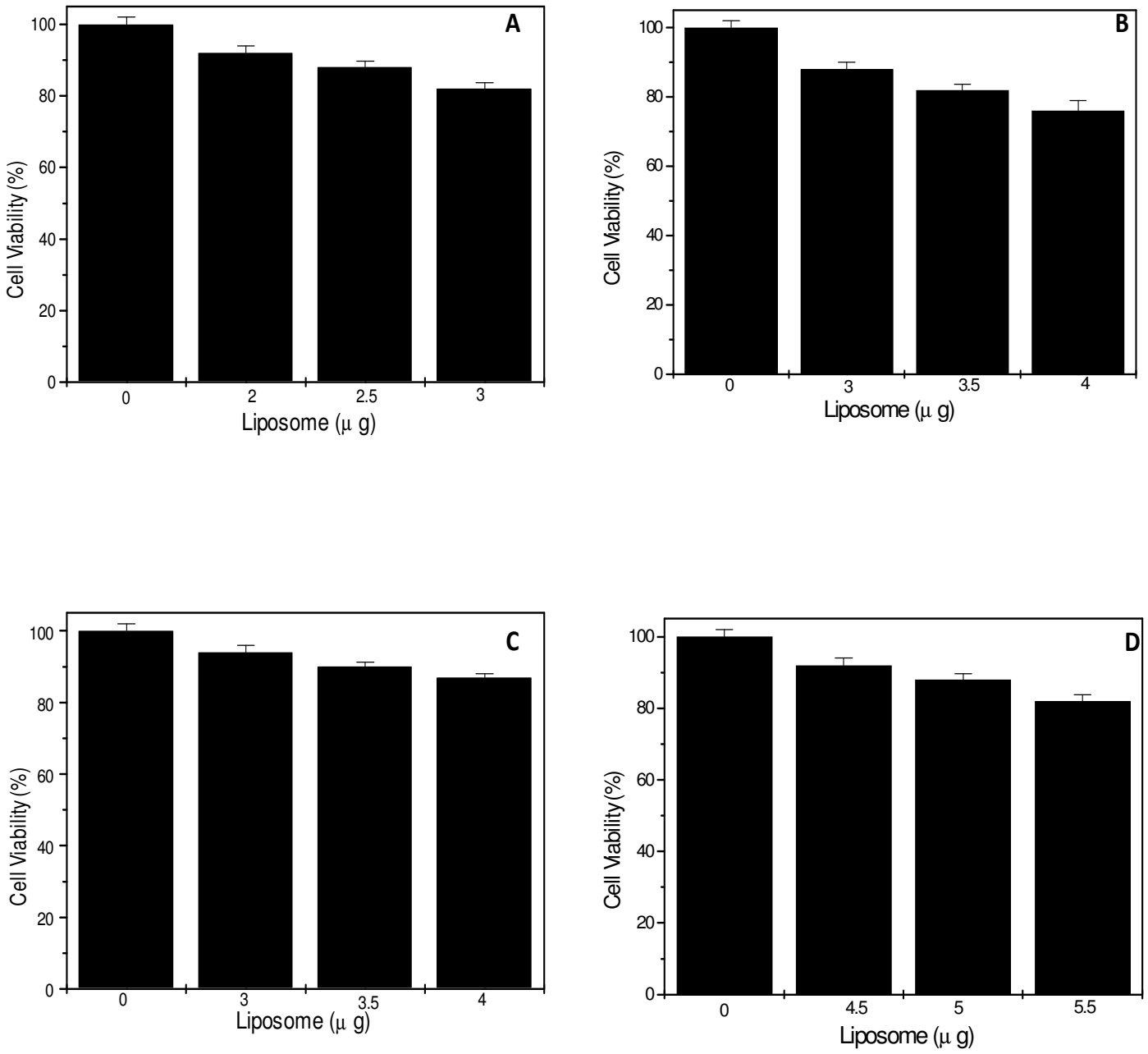

Figure 5. Cytotoxicity assays of pegylated lipoplexes in HEK 293 cells. Assays were conducted in 48 well plates with 0.5 µg pGL3 DNA and increasing amounts of liposomes as indicated. A) 2% pegylated DC-Chol liposomes; B) 5% pegylated DC-Chol liposomes; and C) 2% pegylated Chol-T liposomes; 5% pegylated Chol-T liposomes. Results are presented as means ± S.D. (n = 3).

embedded cholesteryl moieties.

Conclusion

Hence it may be concluded that pegylation of Chol-T and DC-Chol liposomes with PEG_{2000} up to a 5 mole percentage does not alter the previously observed trend that 3C spacer CCCs offer a transfection advantage over their 2C spacer counterparts. Moreover, liposome (+ve) : DNA (-ve) charge ratios at the optimal DNA binding ratio for the four liposomes studied here lie in the range of 1.5:1 to 2.5:1. This is in agreement with findings from other studies with unpegylated CCC liposomes (Piperno-Neumann et al., 2003; Singh and Chaudhuri, 2004; Percot et al., 2004), further supporting the position that pegylation levels selected for this study do not greatly influence the DNA accessibility of CCC head groups on cationic liposomes formulated with near equimolar amounts of DOPE.

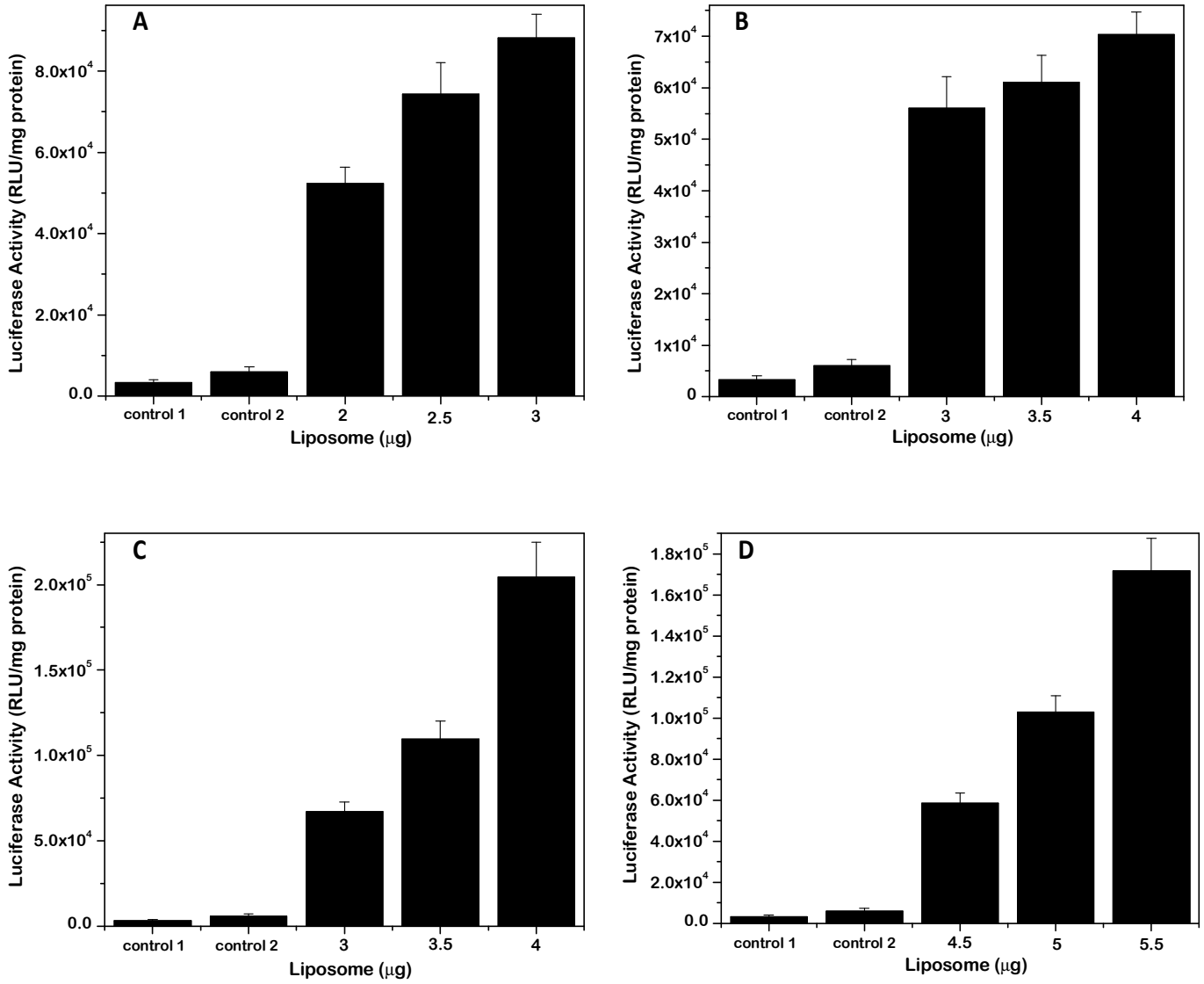

Figure 6. Transfection assays with pegylated lipoplexes in HEK 293 cells. Assays were conducted in 48 well plates with 0.5 µg pGL3 DNA and increasing amounts of liposomes as indicated. A) 2% pegylated DC-Chol liposomes; B) 5% pegylated DC-Chol liposomes; and C) 2% pegylated Chol-T liposomes; 5% pegylated Chol-T liposomes. Results are presented as means ± S.D. (n = 3).

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