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Lipoplexes with biotinylated transferrin accessories: Novel, targeted, serum-tolerant gene carriers

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Abstract

Novel transfecting assemblies comprising biotinylated cationic liposomes, DNA and tribiotinylated transferrin-streptavidin (streptavidin(bio^3-transferrin)) accessories have been prepared, characterized and evaluated for toxicity and DNA delivery capability in human cervical carcinoma cells (HeLa). Two new lipophilic cholesteryl-based biotin derivatives, biotinylcholesterylformylhydrazide (MSB1) and aminohexanoylbiotinylcholesterylformylhydrazide (MSB2) provided docking points for streptavidin(bio^3-transferrin) on cationic liposomes which were formulated with *N*,*N*-dimethylaminopropylaminylsuccinylcholesterylformylhydrazide (MS09) and dioleoylphosphatidylethanolamine (DOPE) in a 2:48:50 molar ratio. Ethidium dye displacement assays and gel retardation studies suggest that in ternary complexes, the DNA is electrostatically bound to the cationic liposomes while transferrins remain liposome-bound through streptavidin–biotin interactions. Assemblies fully protected plasmid DNA from serum nuclease digestion over a range of liposome:pGL3 DNA ratios (3–8:1, w/w) and exhibited low growth inhibition of HeLa cells (circa 5%) at the optimal transfection composition for streptavidin(bio^3-transferrin):liposome:pGL3 DNA of 10:6:1 (w/w/w). Transfection levels, which were twice those of untargeted lipoplexes containing MSB1 or MSB2, were not significantly diminished in the presence of 10% foetal bovine serum. Excess transferrin (200 μg per well) reduced transfection levels to those of untargeted complexes, supporting the notion that at least 50% of ternary complexes gained entry into the cervical carcinoma cells by receptor mediation. Conversely, transfection levels with untargeted lipoplexes were only slightly reduced in the presence of transferrin at the same concentration.

Keywords: Biotin; Transferrin; Cationic liposome; Transfection; Streptavidin

1. Introduction

The rate of entry of lipoplexes into cells varies with cell type and is generally a slow process (Zabner et al., 1995). To speed up this process and to improve the cell specificity of gene expression by a liposome:DNA complex, the incorporation of a homing device into the complex is necessary (Remy et al., 1995; Nishikawa and Huang, 2001). The exploration of the potential of site-specific and targeted drug and gene delivery systems has gained enormous interest recently. The possibility of targeting genes or drugs to specific tissues and cells, as well as facilitating their uptake and cytoplasmic delivery has rendered liposomes a versatile carrier system with numerous potential applications in medicine.

Transferrin is a well-studied ligand for tumour targeting due to the upregulation of transferrin receptors in numerous cancer cell types (Cotten et al., 1990; Wagner et al., 1994; Bellocq et al., 2003). It is also one of the most widely used ligands for synthetic targeting systems. The efficient cellular mechanism of transferrin uptake has been exploited for the delivery of anticancer drugs, proteins, and therapeutic genes into proliferating malignant cells that overexpress transferrin receptors (Singh, 1999). Transferrin can be utilized for targeting either in the form of drug conjugates, hybrid systems with macromolecules or as liposomal-coated systems. The use of transferrin-coupled liposomes for drug (e.g. doxorubicin) delivery has enhanced uptake by cells via receptor mediated endocytosis (Li and Qian, 2002). Other transferrin conjugates such as transferrin-polylysine (Cotten et al., 1990; Wagner et al., 1991; Curiel et al., 1992; Schoeman et al., 1995), transferrin–PEI (Ogris et al., 1999; Kursa et al., 2003) and transferrin–protamine enhanced liposomes (Tros de Ilarduya et al., 2002) have been shown to be efficient carriers for the intro-

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duction of genes into various cells such as human leukemic cells (K-562) and haematopoietic cells. Transferrin–PEI conjugates have been used to efficiently mediate topical gene transfer to the lungs after intratracheal nebulization (Rudolph et al., 2002). Transferrin receptor levels are elevated in various cancer cells, including squamous cell carcinomas such as oral tumours, and seem to correlate with the proliferative ability of tumour cells. Hence, transferrin receptors may be useful as potential targets for drug/gene delivery (Joo and Kim, 2002). HeLa cells used in this study express up to 2×10^5 receptors per cell (Bridges and Smith, 1985), while rat reticulocytes express an average of 85 000 transferrin receptors per cell (Morgan, 1981), and hepatocytes about 18 600 receptors per cell (Bridle et al., 2003).

Avidin and streptavidin, the two biotin-binding proteins, may be targeted to specific tissues when modified with appropriate tissue or cell markers. Their resistance to proteolytic enzymes supports long-term accumulation at the target tissue or organ, and their biotin binding sites permit the delivery of biotinylated molecules or carriers loaded with cytotoxic drugs or other bioactive substances (Chen et al., 2000). Streptavidin is a non-glycosylated 52 800 Da protein with a near-neutral isoelectric point (p*I* 5–6), and exhibits less nonspecific binding than avidin.

Receptor-mediated endocytosis via the transferrin receptor using non-liposomal conjugates of streptavidin or avidin, biotinylated transferrin and biotinylated polylysine with pRSVL DNA has been described previously (Strydom et al., 1993; Schoeman et al., 1995). The addition of two to four PEG chains to the above conjugates appeared to increase luciferase (transgene) activity four- to five-fold in a HeLa cell system (Robinson et al., 1997). Streptavidin has recently been used as a linker between biotinylated polyethyleneglycol–phospholipid present in a liposome bilayer and a monoclonal antibody raised against the rat transferrin receptor.

In this study we outline the synthesis of two novel biotinylated cholesterol derivatives, their formulation with the cationic cholesterol derivative *N*,*N*-dimethylaminopropylaminylsuccinylcholesterylformylhydrazide (MS09) (Singh and Ariatti, 2006) and dioleoylphosphatidylethanolamine (DOPE) to produce liposomes, and the formation of a novel ternary complex between these biotinylated liposomes, plasmid DNA (pGL3) and a streptavidin–biotin–transferrin conjugate (Fig. 1).

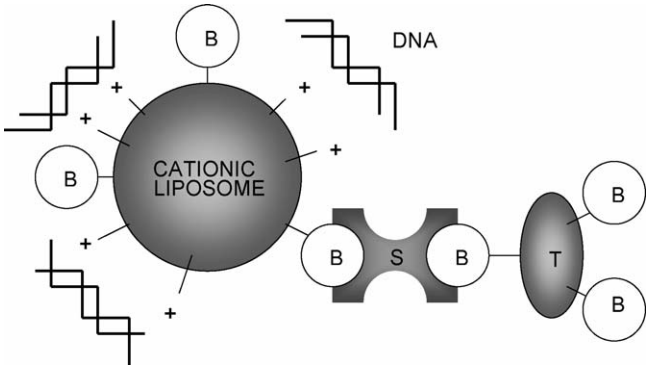

Fig. 1. Model of ternary assembly comprising biotinylated cationic liposome, streptavidin(bio^3-transferrin) and plasmid DNA. B: biotin; S: streptavidin; T: transferrin. Not to scale.

The transferrin ligand (targeting moiety) was first conjugated to three biotin molecules followed by docking to streptavidin to produce a streptavidin–biotin–transferrin (streptavidin(bio^3-transferrin)), complex. The streptavidin in the conjugate acts as a bridge between the bio^3-transferrin and liposome-anchored biotin moieties. Biotinylated lipoplexes and transferrin-conjugated lipoplexes were then characterized, and evaluated for toxicity and transfection potential in HeLa cells.

2. Materials and methods

2.1. Materials

Cholesteryl formylhydrazide and MS09 were prepared as described [32]. Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), biotinamidocaproate-NHS, apotransferrin, ferric citrate, dioleoylphosphatidylethanolamine (DOPE), human serum transferrin, bicinchoninic acid (BCA protein assay kit) and streptavidin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dry pyridine, dimethylformamide (DMF), *p*-dimethylaminocinnamaldehyde, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid (HEPES), glutarylaldehyde, osmium tetroxide, propylene oxide, lead citrate, bromophenol blue, xylene cyanol, uranyl acetate, ethidium bromide, Coomassie blue G250, dimethylsulphoxide (DMSO), crystal violet and silica gel $60F_{254}$ chromatography plates were obtained from Merck (Darmstadt, Germany). The components of Spurr's resin (GRL 4206, DER 736, NSA, S-1), were supplied by TAAB Laboratories (Berkshire, UK). Copper grids for electron microscopy were obtained from Capital Labs (KwaZulu-Natal, South Africa). Formvar-coated grids were prepared by the Electron Microscope Unit, University of KwaZulu-Natal (Pietermaritzburg, South Africa). Bovine serum albumin (BSA) and pBR322 DNA were purchased from Roche Diagnostics (Mannheim, Germany). Sypro Red™ protein stain was purchased from Life Technologies (UK). The pGL3 control vector was obtained from Promega Corporation (Madison, WI, USA) and amplified according to the manufacturer's protocol. Agarose was obtained from Bio-Rad Laboratories (Richmond, CA, USA). Tissue culture plasticware was obtained from Bibby-Sterilin (Staffordshire, UK). All other reagents were of analytical grade. Ultrapure water (Milli-Q50) was used throughout.

2.2. Cell culture

HeLa cells (Highveld Biologicals Pty Ltd., Kelvin, South Africa) were propagated at 37 °C in 25 cm^2 screw cap flasks in 5 ml of minimum essential medium (MEM) with Earle's salts (Gibco-BRL, Life Technologies Ltd., Inchinnan, Scotland) supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), 20 mM HEPES (pH 7.5) and 10% (v/v) heat inactivated foetal bovine serum (Delta Bioproducts, Johannesburg, South Africa). Cultures were routinely trypsinized in 0.25% (w/v) trypsin, 0.1% (w/v) EDTA (Whittaker, M.A. Bioproducts, Maryland, USA) and passaged 1:4 every 4 days. Cell stocks in complete medium containing 10% (v/v) DMSO were frozen and stored in a −80 °C biofreezer.

2.3. Preparation of biotinylcholesterylformylhydrazide (MSB1)

The *N*-hydroxysuccinimide ester of biotin (biotin-NHS) was prepared by an adaptation of the method described by Wilchek and Bayer (1988). Briefly, biotin (122 mg, 0.5 mmol), DCC (99 mg, 0.48 mmol) and NHS (60 mg, 0.52 mmol) were dissolved in 1.2 ml DMF. Dicyclohexylurea crystals were removed by filtration after 6 h and the DMF was evaporated to dryness in a Büchii Rotavapor-R. The residue was extracted three times with ether. The biotin-NHS was recrystallized from isopropanol; mp 198 °C.

Biotin-NHS (68 mg, 0.2 mmol) and cholesterylformylhydrazide (88 mg, 0.2 mmol) were dissolved with gentle heating in 4 ml dry pyridine. The reaction mixture was kept at room temperature in the dark for 48 h and thereafter concentrated in vacuo. The residue was then partitioned in a $CHCl_3$:H_2O (1:1, v/v) mixture. The NHS was removed in the aqueous layer and the product dissolved in the chloroform layer. The $CHCl_3$ layer was evaporated under reduced pressure and the residue was crystallized from ethanol. The product was purified further by preparative TLC on 10 cm × 20 cm silica gel $60F_{254}$ chromatography plates which were developed in $CHCl_3$: MeOH (9:1, v/v) (solvent system A). The MSB1 gave a single spot by TLC (R_f: 0.52) which turned pink/red when sprayed with 0.2% *p*-dimethylaminocinnamaldehyde in 2% sulphuric acid (detection of the biotin group). mp: 192–193 °C. Yield: 80.2 mg (60%). IR (film): 3288 (b, N–H), 2939 (st, C–H), 1695 (st, C–O), 1244 (m, C=N), 1465 (m, C=C), 1045 (m, C–S). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.65 (s, 3H, C–CH_3), 0.88 (d, 3H, J = 6.5, C–CH_3), 0.84 (d, 6H, J = 6.1, C–CH_3), 4.32 (m, 1H, biotin H), 4.48 (m, 2H, biotin H, Chol-$H_{3\alpha}$), 5.35 (bs, 1H, Chol-H_6). MS, *m/z*, ES-TOF: 693.6136 [$M + Na^+$], 694.6306 [$M + H + Na^+$].

2.4. Preparation of aminohexanoylbiotinylcholesterylformylhydrazide (MSB2)

Biotinamidocaproate-NHS (11.5 mg, 0.025 mmol) and cholesterylformylhydrazide (11.25 mg, 0.025 mmoles) were dissolved in 400 μl dry pyridine. The reaction mixture was kept at room temperature in the dark for 48 h and thereafter evaporated to dryness in a rotary evaporator to produce a white film. Distilled water was added to completely cover the product. Free NHS was extracted into the water. The mixture was kept at 4 °C overnight, and the water finally removed by filtration and product dried under vacuum. The product was monitored at each stage by TLC (silica gel $60F_{254}$) in solvent system A. Biotin containing compounds on TLC plates were detected with a *p*-dimethylaminocinnamaldehyde solution (Section 2.3). MSB2 gave a single spot by TLC (R_f: 0.33 in solvent A) with a mp of 194–195 °C and a yield of 17.8 mg (91%). IR (film): 3446 (b, N–H), 2929 (st, C–H), 1669 (st, C=O), 1244 (w, C–N), 1460 (w, C=C), 1032 (m, C–S). MS, *m/z*, ES-TOF: 806.7360 [$M + Na^+$], 807.7563 [$M + H + Na^+$]

2.5. Preparation of bio^3-transferrin

Transferrin was initially iron loaded by an adaptation of the method described by Kursa et al. (2003). Briefly, ferric citrate buffer (25 μl, 10 mM, pH 7.8) was added to transferrin (5 mg) and diluted to 1 ml with 20 mM HEPES, 150 mM NaCl (pH 7.4). Thereafter, bio^3-transferrin was prepared from iron loaded transferring and biotinamidocaproate-NHS as described (Schoeman et al., 1995).

2.6. Preparation of streptavidin(bio^3-transferrin)

Preparation of this complex was carried out as described by Schoeman et al. (1995). Briefly streptavidin (3 mg, 0.05 μmol) was initially dissolved in 2 ml 0.2 M NaCl, 0.005 M Tris–HCl (pH 7.6). To a solution of bio^3-transferrin in water (0.55 ml, 4 mg, 0.05 μmol) was added 1.45 ml 0.2 M NaCl, 0.005 M Tris–HCl (pH 7.6). The streptavidin and bio^3-transferrin solutions were then mixed together quickly and checked for turbidity. This was kept at room temperature for 1 h in the dark. Solutions were re-examined and were clear of any turbidity (Strydom et al., 1993; Schoeman et al., 1995). Complexes were stored frozen at −10 °C.

2.7. Formulation of biotinylated liposomes containing biotinylcholesterylformylhydrazide (MSB1) and aminohexanoylbiotinylcholesterylformylhydrazide (MSB2)

Components for liposome MSB1 and MSB2 were formulated in a 48:50:2 molar ratio of MS09:DOPE:MSB1/MSB2 with DOPE fixed at 2 μmol. Liposome components (4 μmol of lipid) were dissolved in 1 ml $CHCl_3$ and evaporated to a thin film by rotary evaporation. After overnight incubation in vacuo the thin film was rehydrated in 1 ml sterile HEPES buffer overnight (20 mM HEPES, 150 mM NaCl, pH 7.5). The suspension was then vortexed and sonicated for 5 min at 20 °C in a Transonic bath type sonicator. The resulting liposome suspension was stored at 4 °C.

2.8. Characterization of cationic liposomes by transmission electron microscopy

To each liposome suspension (50 μl) was added bovine serum albumin in 0.1 M Tris–HCl buffer (5% (w/v), 100 μl, pH 7.5) followed by 25% glutaraldehyde (50 μl). After 20 min the resultant gels were diced and treated with osmium tetroxide in the dark for 24 h. Thereafter the samples were dehydrated stepwise (70–100% ethanol) and placed successively in propylene oxide (20 min) and propylene oxide:Spurr's resin (1:1, v/v) for another 20 min. Samples were finally placed in pure Spurr's resin for 45 min and embedded in beam capsules in vacuo at 60 °C for 48 h. The resultant blocks were sectioned using a Reichert-Jung ultracut microtome. Sections were collected on C-200 copper grids. Grids were stained with uranyl acetate (10 min) and lead citrate for a further 10 min. Stained sections were viewed using a Jeol 1010 transmission electron microscope at 60 kV. Liposomes

were photographed at a 2 s exposure on a fine grain release positive film.

2.9. Gel retardation studies with pGL3 plasmid DNA

Varying ratios of different biotinylated liposome:DNA complexes and streptavidin(bio^3-transferrin):liposome:DNA assemblies were set up as indicated in Fig. 4. All cationic liposome:DNA complexes were incubated for 30 min at room temperature and subsequently 3 μl of gel loading buffer/stop solution (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol in a 2× gel buffer) was added to all samples. The samples were then loaded onto 1% agarose gels and subjected to electrophoresis for approximately 90 min, at 50 V in a buffer containing 36 mM Tris–HCl, 30 mM sodium phosphate and 10 mM EDTA (pH 7.5). The gel was stained with ethidium bromide (1 μg/ml) for 30 min and viewed under transillumination at 300 nm using a UVP gel documentation system. Images were obtained and photographed at 320 ms exposure. Some gels were stained for protein with a 0.5% Coomassie Blue G250 solution for 20 min before photographing under white light.

2.10. Dye displacement assays

Four different dye displacement assays were carried out to probe liposome:DNA binding interactions and the effect of transferrin and streptavidin(bio^3-transferrin) on binding of DNA to liposomes. All fluorescence measurements were carried out on a Shimadzu RF-551 spectrofluorometer at a wavelength of 600 nm and an excitation wavelength of 520 nm. In each case baseline fluorescence (0%) was established using a solution of ethidium bromide (1 μg) in 500 μl of 20 mM HEPES, 150 mM NaCl, pH 7.5.

To probe liposome–DNA interactions 6 μg of pBR322 DNA was added to the baseline solution and the new reading was assumed to represent 100% fluorescence. Aliquots of each biotinylated liposome, MSB1 or MSB2 (6 μg, 2.2 μl), were added stepwise to the solution until 60 μg of each liposome had been added. In this and subsequent assays the solutions were mixed thoroughly after each addition before recording fluorescence values. Results were plotted relative to 100% fluorescence.

To explore DNA interaction with liposome:transferrin (1:2) mixtures, baseline (0%) and 100% fluorescence values were established as described above. Aliquots (3 μl) of each liposome:transferrin complex (3.9 μg lipid and 7.8 μg transferrin) were added stepwise to the DNA–ethidium bromide solution until the total lipid amounted to 39 μg and the total transferrin was 78 μg.

The effect of streptavidin(bio^3-transferrin) adducts on lipoplexes was next examined. Biotinylated liposome:DNA lipoplex (6:1, w/w, 36 μg lipid and 6 μg pBR322 DNA) was added to the baseline solution and the fluorescence reading recorded as arbitrary fluorescence units. Aliquots (2 μl, 1.74 μg) of the streptavidin(bio^3-transferrin) complex were added stepwise to the solution until 26.1 μg of the streptavidin(bio^3-transferrin) had been added.

To examine the effect of iron-loaded transferring on lipoplexes a biotinylated liposome:DNA mixture (6:1, w/w, 36 μg lipid and 6 μg pBR322 DNA) was added to the baseline solution and the fluorescence reading recorded and reported as arbitrary fluorescence units. Aliquots (2 μl, 4 μg) of iron loaded transferrin were added stepwise to the solution until a total of 28 μg of transferrin had been added.

2.11. Transmission electron microscopy of lipoplexes (MSB1 and MSB2) and liposome:DNA:streptavidin(bio^3-transferrin) ternary complexes

To 50 μl of the respective lipoplex suspension on parafilm was added 0.5% uranyl acetate (50 μl). This was mixed and allowed to stand for 3 min. The matt surface of formvar coated grids, were brought into contact with the lipoplex–uranyl acetate mixture for 3 min. Thereafter discs were air dried overnight and viewed in a Jeol 1010 transmission electron microscope at 60 kV.

2.12. Nuclease digestion assays of ternary complexes

Mixtures of pGL3 DNA (1 μg), streptavidin(bio3-transferrin) (10 μg) and liposomes (3–8 μg) in 20 μl with 20 mM HEPES, 150 mM NaCl, pH 7.5 were prepared by adding streptavidin(bio^3-transferrin) complexes to liposomes and mixing prior to the addition of DNA. Following a 30 min incubation at 21 °C fetal bovine serum was added to complexes to a final serum concentration of 10% (v/v). Samples were then incubated at 37 °C for 4 h. Thereafter EDTA was added to a final concentration of 10 mM and sodium dodecyl sulphate (SDS) to a final concentration of 0.5% (w/v). The reaction mixtures were incubated further at 55 °C for 20 min after which samples were subjected to agarose gel electrophoresis as described (Section 2.9).

2.13. Growth inhibition assays

HeLa cells were trypsinized and seeded into a 24-well plate at a seeding density of 2.2×10^4 cells per well for both MSB1:DNA and MSB2:DNA lipoplexes. The HeLa cell seeding density was 2.1×10^4 cells per well for both MSB1 and MSB2 streptavidin(bio^3-transferrin):liposome:DNA ternary complexes. Cells were then incubated at 37 °C for 24–36 h to achieve semi-confluency. Lipoplexes and targeted ternary complex mixtures were diluted to 10 and 20 μl, respectively, with 20 mM HEPES, 150 mM sodium chloride, pH 7.5. Cells were prepared by first removing the growth medium and replacing it with 0.5 ml of serum-free medium. The reaction complexes were then added to wells. The cells were then incubated at 37 °C for 4 h. Thereafter, the medium was replaced with complete medium (MEM + 10% FBS + antibiotics) and cells were incubated at 37 °C for a further 48 h. The cells were then quantified by the method of Schellekens and Stitz (1980). In brief, cells were washed twice with PBS and stained with 200 μl crystal violet solution (0.5% (w/v) crystal violet, 0.8% (w/v) sodium chloride, 5% (v/v) formaldehyde, 50% (v/v) ethanol) for 20 min. The

stain was then removed and the cells washed extensively with water. The multiwell plate was then dried for 24 h, and the stain extracted with 2-methoxyethanol (0.5 ml) over a period of 36 h, with gentle rocking (20 rpm) on a Stuart Scientific STR 6 platform shaker. Absorbance values for the samples were then read in a Novospec spectrophotometer, at a wavelength of 550 nm.

2.14. Transfection studies with untargeted and targeted biotinylated liposomes

Transfections by lipoplexes were conducted on the HeLa cell line in the presence and absence of 10% FCS. Competition assays with iron loaded transferrin were also conducted. HeLa cells were trypsinized and seeded into a 24-well plate at seeding densities of 2.5–2.9 × 10^4 cells per well for studies conducted in the absence and presence of 10% FBS and competition assays. The cells were allowed time to attach to the wells and to grow to semi-confluency (24–36 h). Transfection complexes were incubated at 21 °C for 30 min prior to addition to cells. The cells were prepared by removal of growth medium, washing with 0.5 ml PBS and replenishment with 0.5 ml serum-free medium (MEM + antibiotics). For the evaluation of transfection efficiency in the presence of serum the medium utilized was complete medium (MEM + antibiotics + 10% FBS). Competition assays utilizing 200 μg free iron loaded transferrin per well were also conducted. The iron loaded transferrin was added to the cells 10 min prior to the addition of untargeted or targeted biotinylated lipoplexes. Following the addition of lipoplexes, cells were incubated at 37 °C for 4 h, after which the medium was removed and replaced with complete growth medium (MEM + antibiotics + 10% FBS). Cells were incubated for a further 48 h at 37 °C after which they were assayed for luciferase activity using the luciferase assay system (Promega). The soluble protein in cell free extracts was determined using the BCA assay (Sigma) with BSA as the standard protein.

3. Results and discussion

3.1. Chemical synthesis and protein modifications

Two novel biotinylated cholesterol derivatives were synthesized (Fig. 2), by the condensation of *N*-hydroxysuccinimide activated biotinyl components and cholesterylformylhydrazide. Both derivatives have a common cholesterol anchor, but differ in the lengths of their spacer arms, with MSB2 having the longer spacer arm (Fig. 2b). Structures of MSB1 and MSB2 were confirmed by infrared (IR) spectrophotometry on a Nicolet Impact 420 spectrophotometer, ^{1}H (300 MHz) NMR on a Gemini 300 instrument and electrospray time of flight mass spectrometry on a Waters APIQ-TOF Ultima instrument. Both compounds were used in the formulation of two novel biotinylated liposomes together with MS09 and DOPE, which were assessed for their transfection activity in the HeLa cell line with and without transferrin as the targeting moiety. Iron (Fe^{3+}) loaded apotransferrin binds strongly to the transferrin receptor and was used in all experiments requiring transferrin. In the streptavidin(bio^3-transferrin) complex three aminohexanoylbiotin moieties are attached to transferrin. The biotin content of transferrin is generally kept low to reduce cross-linking and precipitation of complexes under normal buffer incubation conditions (Hawtrey and Ariatti, 1999) and to minimize structural perturbations of the transferrin (Schoeman et al., 1995). This transferrin receptor-targeted complex was linked via the streptavidin component to the biotinylated liposomes (Fig. 1).

3.2. Preparation and characterization of liposomes

Liposomes were formulated to contain a cationic component for interaction with the plasmid DNA, a biotinylated cholesterol derivative intended for interaction with streptavidin in

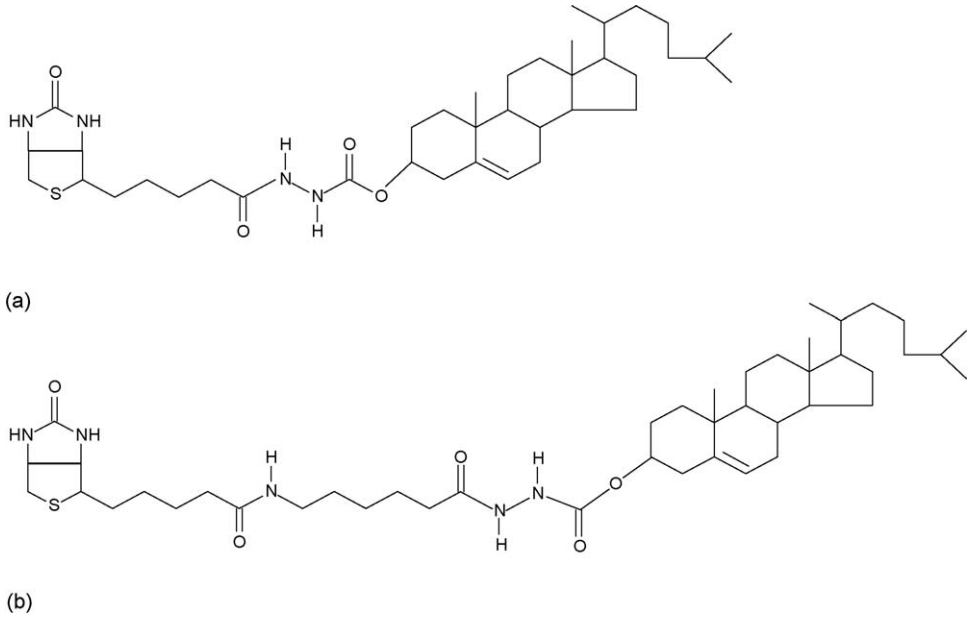

Fig. 2. Structure of (a) Biotinylcholesterylformylhydrazide (MSB1), and (b) aminohexanoylbiotinylcholesterylformylhydrazide (MSB2).

the streptavidin(bio^3-transferrin) complex, and a helper lipid, DOPE, that plays a role in increasing transfection efficiency. Liposomal suspensions, which were prepared as described (Section 2.7) were stable at 4 °C over several weeks and other lipid ratios were not explored. Transmission electron microscopy confirmed the unilamellar nature of vesicles. Liposome MSB1 vesicles were found to be in the 50–200 nm size range (Fig. 3a), whilst MSB2 liposomes ranged from 100 to 300 nm in diameter (Fig. 3b). It has been proposed that the liposome diameters or hydrodynamic radii and their population dispersity bear a direct relationship to liposome clearance rates in blood and can exert marked effects on the antitumour activity of therapeutic agents (Campbell et al., 2001). The size of liposomes and the rigidity of the liposomal membrane are also important factors for liposome removal by the reticuloendothelial system (RES). Small liposomes with less fluid membranes have longer half lives in the blood stream and are not easily opsonized by complement factors in the blood when compared to larger liposomes. Liposome sizes of 100–200 nm are also considered to be favourable for tumour targeting (Oku et al., 2000).

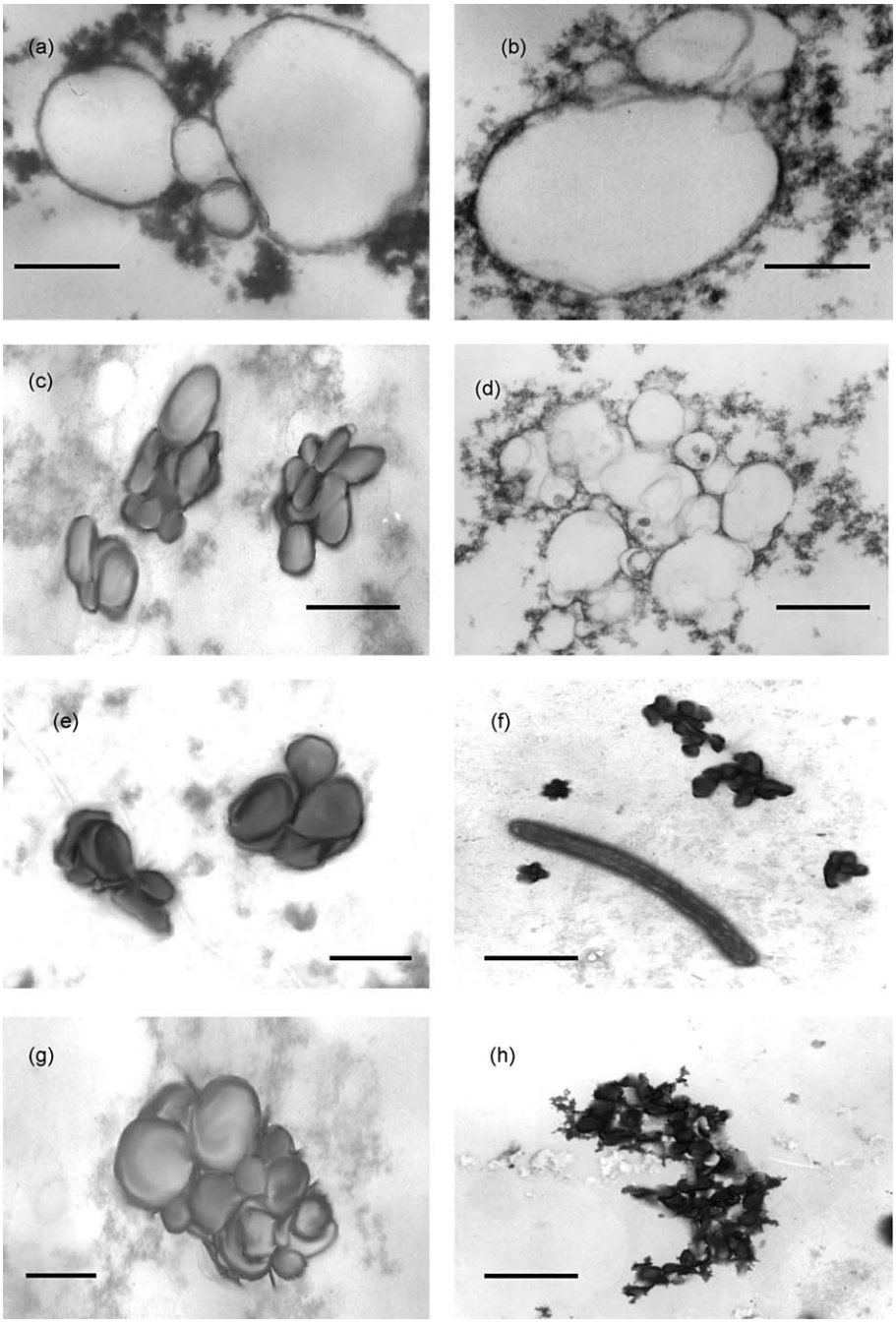

Fig. 3. Transmission electron micrographs of liposomes, lipoplexes and ternary assemblies. Bar = 100 nm except (f) (500 nm). (a) MSB1 liposomes; (b) MSB2 liposomes; (c) MSB1:DNA lipoplexes, 6:1 (w/w) ratio (1.4:1, +/− charge ratio); (d) MSB2:DNA lipoplexes, 6:1 (w/w) ratio (1.4:1, +/− charge ratio); (e) streptavidin(bio^3-transferrin):MSB1 liposome:DNA, 10:6:1 (w/w/w), (f) 10:7:1 (w/w/w); (g) streptavidin(bio^3-transferrin):MSB2 liposome:DNA, 10:6:1 (w/w/w), (h) 10:7:1 (w/w/w). Bar = 100 nm except (f) (500 nm).

3.3. Lipoplex and ternary complex formation

Lipoplexes were generated by the addition of DNA to liposomes followed by an incubation at 21 °C for 30 min. The ternary complexes (streptavidin(bio^3-transferrin):liposome:DNA) were prepared by first adding the streptavidin(bio^3-transferrin) to the liposome followed by the DNA, allowing 15 min incubation after the addition of each component. All the complexes were characterized by gel retardation assays, dye displacement assays, nuclease digestion assays and transmission electron microscopy. It was reported previously that the formation of complexes in which ferritin molecules had been conjugated via streptavidin to biotinylated liposomes can be impaired by unfavourable electrostatic repulsion. The charge of the liposomes was therefore adjusted by the addition of a cationic surfactant in the lipid bilayer (Velev, 1997). Liposomes used in the present study also contain a cationic cholesterol derivative (MS09) which afforded very effective lipoplexes (Singh and Ariatti, 2006). The liposomes become coated with the transferrin ligands for specific interaction with the cognate cell surface receptors thereby transporting the plasmid DNA that is bound to the cationic component of the liposome.

3.4. Gel retardation studies

Gel retardation studies were conducted on both lipoplexes (MSB1 and MSB2). It can be seen in Fig. 4a and b that as the amount of cationic liposome was increased, more DNA was bound electrostatically to the cationic liposome and hence less DNA entered the gel. Complete retardation of the DNA in the case of each liposome preparation was obtained at the same biotinylated liposome:DNA binding ratios, viz., 6:1 (w/w). Complete retardation normally indicates that the negative charges of the DNA have been completely titrated by the cationic liposomes' positive charges. This ratio was then used in defining the ratio of components in ternary complexes with streptavidin(bio^3-transferrin) that would afford maximal transfection activity. By retaining this liposome:DNA ratio in ternary complexes, it was reasoned that ionic interaction between streptavidin(bio^3-transferrin) and the cationic components of liposomes would be minimized thus favouring the biotin–streptavidin interaction as the docking force.

3.4.1. Transferrin:DNA complexes and ternary assemblies

It is clear, from the results presented in Fig. 4c, that there is little if any binding of transferrin to DNA (1 μg) over a wide protein range at pH 7.5. Hence, ternary complexes, that are formed in this study, are held together largely by the biotin–streptavidin interaction and ionic forces (liposome and DNA). It has been suggested, using DOTAP/DOPE liposomes, that transferrin binds to vesicles through negatively charged groups on this ligand, at physiological pH, to afford complexes which bind DNA through a charge–charge interaction (Tros de Ilarduya and Düzgüneş, 2000). The lack of transferrin affinity for DNA (Fig. 4c) may be attributed, in part, to the fact that the transferrin molecule bears four negatively charged sialic acid residues (Nagaoka and Maitani, 2001), and hence is an acidic protein, with the diferric species having an isoelectric point (p*I*) in the range 5.6–5.8 (Qian et al., 2002). It has previously been reported that transferrin fails to interact with DNA as determined in a band shift assay (Huckett et al., 1986). However, Joshee et al. (2002) have suggested that transferrin can interact with DNA to give a relatively stable complex. Based on evidence provided by electron microscopy, they have also proposed that a complex comprising DNA (1 μg) and transferrin (20 μg) adopts an unusual toroidal ring structure (diameter: 160 nm) and a tail. Furthermore, the thickness of the DNA component was significantly greater than that of uncomplexed DNA.

At low streptavidin(bio^3-transferrin):liposome ratios the streptavidin(bio^3-transferrin) may bind to the liposomes by charge–charge interactions which precludes binding of the DNA (Fig. 4d (lane 2) and e (lanes 2 and 3)). However, as the streptavidin(bio^3-transferrin) concentration increases it appears that the biotin–streptavidin interaction with the liposomes becomes the dominant cohesive force and DNA is able to successfully compete for binding to the cationic bilayer. The results show that the complete retardation of the ternary complexes for both liposome formulations was achieved at a streptavidin(bio^3-transferrin):liposome:DNA ratio of 10:6:1 (w/w/w, Fig. 4d and e). The determination of this ratio was critical as it established the optimal ratio of the ternary complex to be utilized for transfection purposes. In separate binding studies the liposome amounts were varied from 0 to 8 μg while the streptavidin(bio^3-transferrin) was kept constant at 10 μg and the pGL3 DNA maintained at 1 μg. Gels were stained with Coomassie Blue to detect the protein component of complexes. It may be inferred from Fig. 4f, where the protein is seen to co-localize with the liposomes in the wells therefore, that at least 3 μg or perhaps 4 μg of MSB1 liposomes are required to bind 10 μg of streptavidin(bio^3-transferrin). Similar results were obtained for MSB2 liposomes (data not shown). Protein staining of gels with Sypro Red™ gave the same results (not shown). These findings serve to affirm the ratios of components that have been selected for the transfection studies.

3.5. Dye displacement assays

Assays in which aliquots of biotinylated liposomes were added to plasmid DNA in the presence of ethidium bromide revealed a decrease in ethidium fluorescence (ethidium displacement) until a point of inflection was attained at a liposome:DNA ratio (6:1, w/w) which correlated with the ratio that achieved total retardation of DNA in gel retardation assays (Fig. 5a) corresponding to a charge (+/−) ratio of 1.4:1. The reduction in fluorescence (45–50%) was less than that obtained for non-biotinylated MS09 liposomes (60%) (Singh and Ariatti, 2006). This may be ascribed to perturbations resulting from the added biotinylated cholesterol components (2% (mol/mol) basis) that are also present in the liposomal bilayer. The addition of the liposome:transferrin complex to DNA (Fig. 5b), resulted in a rapid decrease in the fluorescence which stabilized at a liposome:transferrin:DNA ratio of about 12:6:1 (w/w/w) for both liposome formulations. Results presented in Fig. 5b confirm that the plasmid DNA is able to displace the transferrin, as the dye

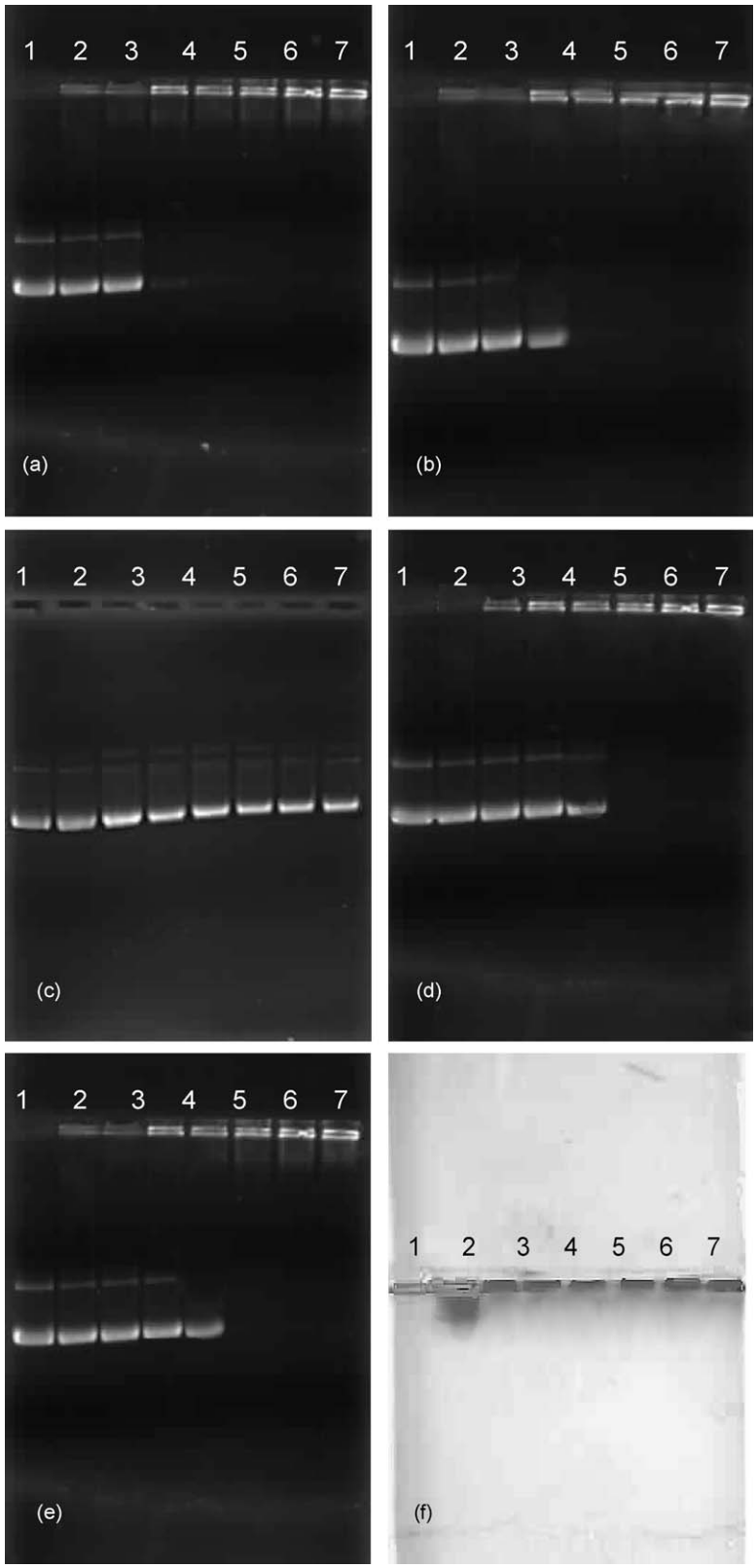

Fig. 4. Gel retardation study of (a) MSB1and (b) MSB2 lipoplexes; (c) transferrin:DNA interaction; (d) and (f) transferrin MSB1 ternary complexes; (e) transferrin MSB2 ternary complexes. (a) and (b) Reaction mixtures (10 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1–8 (0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 μg), while the pGL3 DNA was kept constant at 0.5 μg per well. (c) Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of transferrin in lanes 1–8 (0, 10, 20, 40, 60, 70, 80, 100 μg), while the pGL3 DNA was kept constant at 1.0 μg. (d) and (e) Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1–8 (0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 μg) and pGL3 DNA (1.0 μg), the streptavidin(bio^3-transferrin) was also varied (0, 6, 7, 8, 9, 10, 11, 12 μg). (f) Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1–8 (0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 μg), while the pGL3 DNA and streptavidin(bio^3-transferrin) was kept constant at 1.0 and 10 μg, respectively.

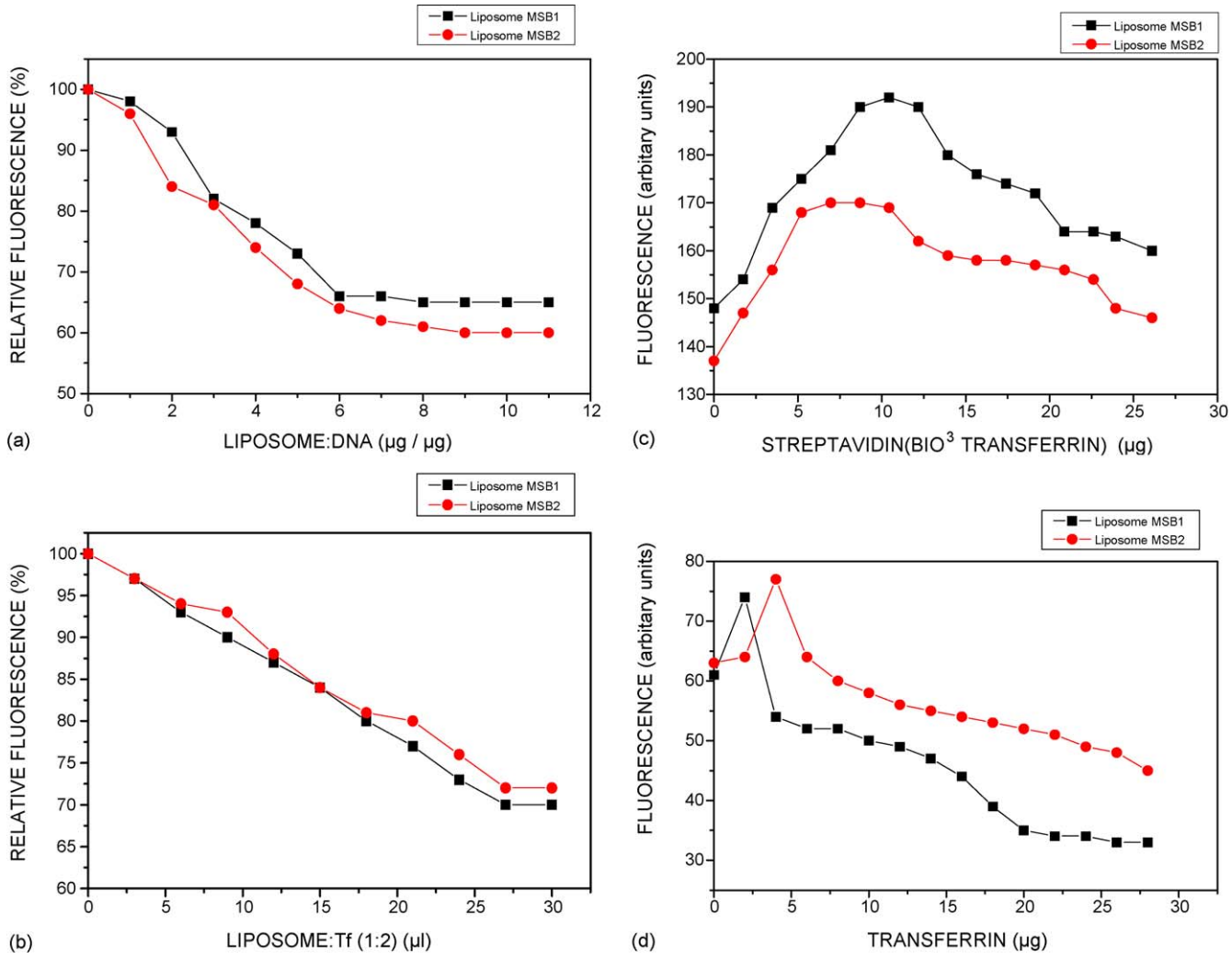

Fig. 5. Ethidium bromide (EtBr) intercalation/displacement assays. All incubation mixtures (500 μl) contained EtBr (1 μg) and pBR322 plasmid DNA (6 μg), 20 mM HEPES and 150 mM NaCl, pH 7.5. (a) Liposomes were added in 6 μg aliquots to a total of 60 μg. (b) Aliquots of liposome:transferrin (1:2, w/w) were added stepwise to a total transferrin of 60 μg. (c) Preformed lipoplexes (liposome:DNA, 36:6, μg/μg) were challenged with increasing amounts of streptavidin(bio^3-transferrin) up to 26.1 μg. (d) To preformed lipoplexes were added increasing aliquots of iron loaded transferrin to a total of 28 μg.

displacement ceases at a liposome:DNA ratio of 6:1 (w/w), the same value achieved in the absence of transferrin. In a separate study, the biotinylated liposome:DNA lipoplex (6:1 w/w) was first added to the ethidium bromide solution prior to addition of the streptavidin(bio^3-transferrin) complex. There was an initial gradual increase in fluorescence upon addition of the streptavidin(bio^3-transferrin) complex (Fig. 5c) which may be due to the initial binding of the streptavidin(bio^3-transferrin) complex to the biotinylated liposomes. This may have interfered with the binding of the DNA to the cationic component of the liposome, thereby partly releasing the DNA from the liposome leading to enhanced fluorescence. The increase in fluorescence observed was more pronounced for liposome MSB1 than for MSB2, suggesting that the liposome MSB2 which has a longer spacer offered less hindrance to the DNA bound to the liposome. This effect was observed up to 6 μg of streptavidin(bio^3-transferrin). At higher concentrations of streptavidin(bio^3-transferrin) there was a gradual quenching in the fluorescence which apparently marked the reassociation of DNA with the liposome complex. It seems, therefore, plausible to suggest that the ionic interaction between the liposomes and streptavidin(bio^3-transferrin) assumes minor importance and that the modified transferrin remains liposome-bound largely through the biotin–streptavidin interaction. This is corroborated by the gel retardation studies which show a strong interaction of streptavidin(bio^3-transferrin) with liposomes that are also associated with a saturating amount of DNA (Fig. 4d–f).

On addition of iron-loaded transferrin (holotransferrin) to the liposome:DNA complex (Fig. 5d), an initial marked increase followed by a sudden drop in the measured fluorescence was noted signaling a binding of the transferrin to the liposome with attendant partial displacement of the DNA in the process. This initial binding of transferrin is immediately followed by a sharp drop in fluorescence to levels below that of the initial reading, supporting the notion that partially displaced DNA becomes reattached and more compacted with increasing iron loaded transferrin. It is noteworthy that in these experiments liposomes and plasmid DNA were premixed at a ratio of 6:1 (w/w), which results in

complete retardation of DNA in a band shift assay (Fig. 4a and b), and marks the saturation point for binding of the DNA to the liposomes in the dye displacement assay (Fig. 5a).

3.6. Transmission electron microscopy of complexes

3.6.1. Liposome:DNA complexes (lipoplexes)

Lipoplexes were assembled from MSB1 and MSB2 cationic liposomes and plasmid DNA. After mixing the lipidic and nucleic acid components, a 30 min period at room temperature was allowed for maturation before samples were taken for TEM. The lipoplexes observed were very similar to those reported for the related MS09-containing lipoplexes formulated without MSB1 and MSB2 lipobiotin derivatives (Singh and Ariatti, 2006). The size and ultrastrucuture of these lipoplexes were observed at their optimal transfection ratio of liposome:DNA (6:1, w/w) and charge (+/−) ratio of 1.4:1. Lipoplex sizes ranged from 100 to 200 nm for MSB1 lipoplexes and 100–300 nm for MSB2 lipoplexes and appeared as aggregates or clusters (Fig. 3c and d). Studies have shown that highly positively charged complexes, where DNA is completely condensed, or highly negatively charged complexes with an excess of DNA over the lipid, exhibit a size distribution between 100 and 450 nm. Neutral complexes however are characterized by a heterogeneous size distribution from about 300 to 1200 nm in diameter with a much lower colloidal stability (Pedroso de Lima et al., 2001).

Lipoplex size is a potentially important factor in determining the success of synthetic gene delivery systems. DNA complexes can range from 50 to 1000 nm depending on DNA:carrier ratio, total concentrations, ionic strength of buffer, and kinetics of mixing, with the lower size limit not easily adjustable and hence intrinsic to that specific formulation (Schätzlein, 2003).

3.6.2. Streptavidin(bio^3-transferrin):liposome:DNA complexes

The ternary complexes which were formed varied in size from 100 to 250 nm for complexes with MSB1 at a streptavidin(bio^3-transferrin):liposome MSB1:DNA ratio of 10:6:1 (w/w/w) (Fig. 3e), and 200–500 nm at a higher ratio of 10:7:1 (w/w/w) (Fig. 3f). The complexes for liposome MSB2 varied in size from 150 to 300 nm at a ratio of 10:6:1 (w/w/w) for the streptavidin(bio^3-transferrin):liposome MSB2:DNA complexes (Fig. 3g), and from 200 to 500 nm at a higher ratio of 10:7:1 (Fig. 3h). The ultrastructure of these complexes did not markedly differ from that of their corresponding lipoplexes (Fig. 3c and d), in that they formed clusters of varying sizes. Rod-like or elongated tubular forms, which were visible (Fig. 3f) at the higher charge ratio, could be due to the fusion of lipid membranes during formation of the ternary complex which completely covers the DNA. Such tubular or rod shaped structures have been observed previously for some lipoplexes (Gershon et al., 1993; Chiruvolu et al., 1994). Tubules were reported to be about 1 μm in diameter and up to 100 μm in length and most of them appeared entangled with each other (Chiruvolu et al., 1994). However, this was not the case for lipoplexes prepared in the present study and rods seemed to be considerably shorter and occurred singly. The sizes of tubular structures were approximately 0.25 μm in diameter and about 4 μm in length (Fig. 3f). Rod-like structures were also observed at the higher charge (+/−) ratio of 3:1 (results not shown). It has been suggested that these unusual structures are formed above the critical ratio and are complexes where the DNA molecules are packed and completely encapsulated within a smooth lipid bilayer (Gershon et al., 1993). The liposome:DNA charge ratio at which tubular formations were observed in the present study

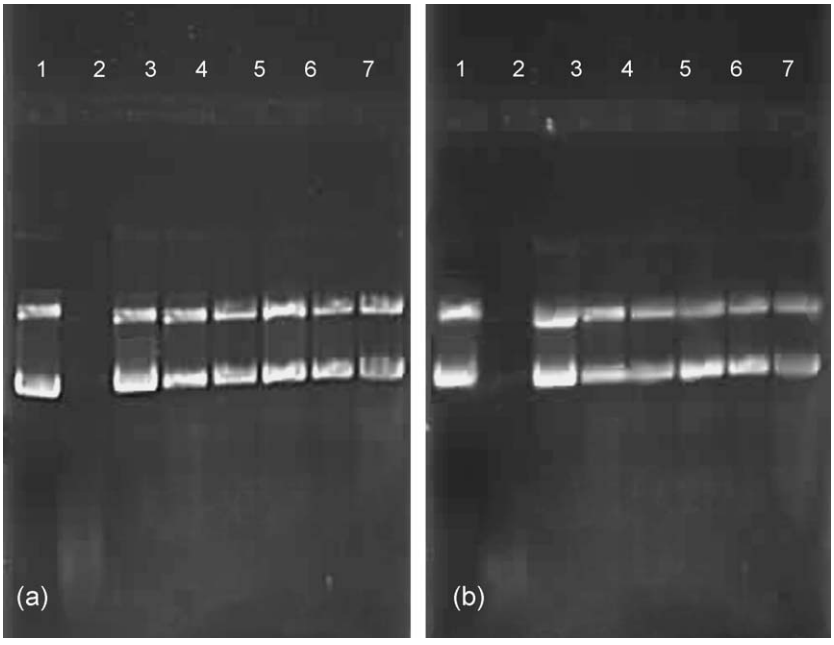

Fig. 6. Nuclease protection assay of ternary assemblies containing: (a) MSB1, pBR322 DNA and streptavidin(bio^3-transferrin), and (b) MSB2, pBR322 DNA and streptavidin(bio^3-transferrin). (Lane 1) Untreated marker plasmid pBR322 DNA (1 μg); (lane 2) unprotected plasmid DNA (1 μg) in the presence of 10% FCS; (lanes 3–8) varying amounts of cationic liposomes (2, 3, 4, 5, 6, 7 μg) with pBR322 DNA (1 μg) and streptavidin(bio^3-transferrin) (10 μg) and 10% FCS.

was 1.6:1. Furthermore, some free liposomes were noted, unlike the preparations of plain lipoplexes, where it appeared that all the liposomes were complex-associated. It has been reported by some investigators that the inclusion of transferrin in the formulation to produce a ternary complex, increased the size of the complex especially if the DNA is added last (Joshee et al., 2002). Lipoplexes reported herein were prepared by the addition of the transferrin component first followed by the DNA, and no prolonged maturation of the complexes (i.e. >30 min) was carried out. Transferrin (80 kDa) itself, is approximately two orders of magnitude smaller than the average lipoplex (200 nm) which can be accommodated in clathrin-coated vesicles. It has been suggested that clathrin-mediated endocytosis of particles exceeding diameters of 200 nm is possible and that the process may involve a considerable degree of size flexibility of the coated vesicles (Zuhorn et al., 2002). The attachment of transferrin to liposomes tends to increase the hydrodynamic radius of the transferrin:liposome:DNA complex (Joo and Kim, 2002). Ideally, for successful endocytosis, the size of the ligand must not exceed the internal diameter of the coated pit.

It has been reported that ternary complexes of 400–500 nm in diameter are appropriate for transfection (Tokunaga et al., 2004). The average size of the most effective ternary complex in vitro utilizing Lipofectin^{TM} (transferrin:liposome:DNA), as determined by light scattering was between 500 and 880 nm, and complex sizes ranged from 400 to1400 nm (Joshee et al., 2002). These complexes do not completely resemble the condensed lipoplexes seen by Gershon et al. (1993) suggesting that uncondensed DNA can also yield high transfection efficiency in liposome-based gene transfer systems. This is at variance with the molecular conjugate based vectors where small vector sizes (up to 17 nm) produced high transfection rates (Perales et al., 1994).

3.7. Nuclease digestion assays of ternary complexes

These studies have shown that the ternary complexes effectively protected the plasmid DNA from attack by the serum nucleases. This could be due to the stability of these complexes or to their compaction by condensation. This is reinforced by the finding that ternary complexes seen under electron microscopy are highly condensed supramolecular structures. Hence, in Fig. 6a and b naked DNA (lane2), is completely degraded by the presence of serum nucleases whereas the DNA bound in the ternary complexes is protected and undegraded, over a range of streptavidin(bio^3-transferrin):liposome:DNA ratios. It has been observed by some investigators that ternary complexes prepared using streptavidin complexes seem to stabilize and protect the DNA from digestion by DNases (Xu et al., 1998). This resonates with the protection of the DNA against nuclease assault revealed by agarose gel electrophoresis in the present study (Fig. 6). It has been noted in DNA/transferrin/PEI complexes that aggregates of the complexes were produced upon addition of serum, due to binding of serum proteins (Ogris et al., 1999). However, ternary complexes in this study showed no aggregation or precipitation upon addition of serum and there was no noticeable change in the turbidity of the liposomal suspension.

3.8. Growth inhibition assays

These studies were conducted on the untargeted biotinylated liposome:DNA complexes and on the ternary complexes for both liposome preparations (MSB1 and MSB2). As can be seen in Fig. 7a and b there was a small but measurable difference in the growth inhibition levels for the plain lipoplexes and the ternary targeted complexes. The ternary complexes showed less cytotoxicity than the plain lipoplexes even at higher liposome ratios. This could be attributed to the presence of the streptavidin(bio^3-transferrin) component. Maximum growth inhibition recorded was about 30% for liposome MSB1:DNA lipoplexes (Fig. 7a) at a liposome:DNA ratio of 8:1 (w/w) corresponding to a +/− charge ratio of 2:1. This result was mirrored by MSB2:DNA lipoplexes (Fig. 7b). Liposome:DNA complexes at a ratio of 4:1 (w/w) or (+/−) charge ratio of 1.0:1, were least inhibitory (about 90% of control). However, maxi-

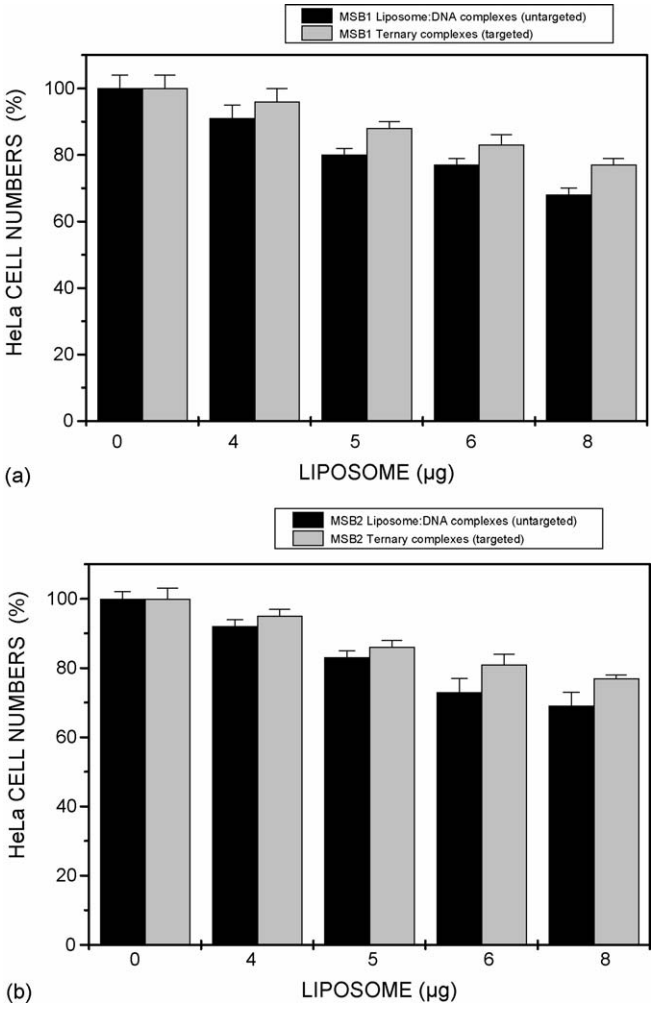

Fig. 7. Growth inhibition studies of lipoplexes and ternary complexes (streptavidin(bio^3-transferrin):liposome:DNA) to the HeLa cell line in vitro. The amount of liposome was varied (0, 4, 5, 6, 8 μg) while the DNA (1 μg) and streptavidin(bio^3-transferrin) (10 μg) was kept constant in a total volume of 0.5 ml MEM. A control sample (no liposome), containing only cells was assumed to have 100% survival. (a) MSB1 Lipoplexes and ternary assemblies; (b) MSB2 lipoplexes and assemblies. Data are presented as mean ± S.D. ($n = 4$).

mum inhibition by ternary complexes (23%) was achieved at a streptavidin(bio^3-transferrin):liposome:DNA ratio of 10:8:1 (Fig. 7a and b). At the lower ternary complex ratio of 10:4:1 the survival rate was about 95% for both MSB1 and MSB2 complexes. The extent of cytotoxicity varies with different liposome formulation and also with the type of complexes being formed with the liposomes. The general trend that the level of toxicity increases with increasing amounts of liposome is also observed for these liposomes although cytotoxicity at the optimal transfecting ratios was low.

3.9. Transfection studies with untargeted and targeted biotinylated liposomes

Transferrin is regarded as a potent ligand that affords effective receptor-mediated gene transfer (Rudolph et al., 2002). Transferrin-facilitated lipofection has therefore been investigated by many researchers who have shown that it occurs with high efficiency (Cheng, 1996; Simões et al., 1998; Joshee et al., 2002; Tros de Ilarduya et al., 2002; Joo and Kim, 2002). It has been shown that mixing of the transferrin component with liposomes prior to DNA addition, as has been the case in this study, prevents DNA condensation and facilitates endocytosis and nuclear targeting thus giving the most effective ligand-directed gene delivery (Joshee et al., 2002).

The transferrin:liposome:DNA complexes (Fig. 8c and d) showed a two-fold increase in transfection activity compared to unconjugated biotinylated liposomes and a threefold increase over plain unbiotinylated MS09:DOPE lipoplexes with the same charge ratio (1.4:1) reported elsewhere (Singh and Ariatti, 2006). This suggests that the presence of the transferrin component facilitates the uptake of the transferrin:liposome:DNA complex into the HeLa cells by receptor mediation. These results are in accordance with findings in a related study (Tros de Ilarduya and

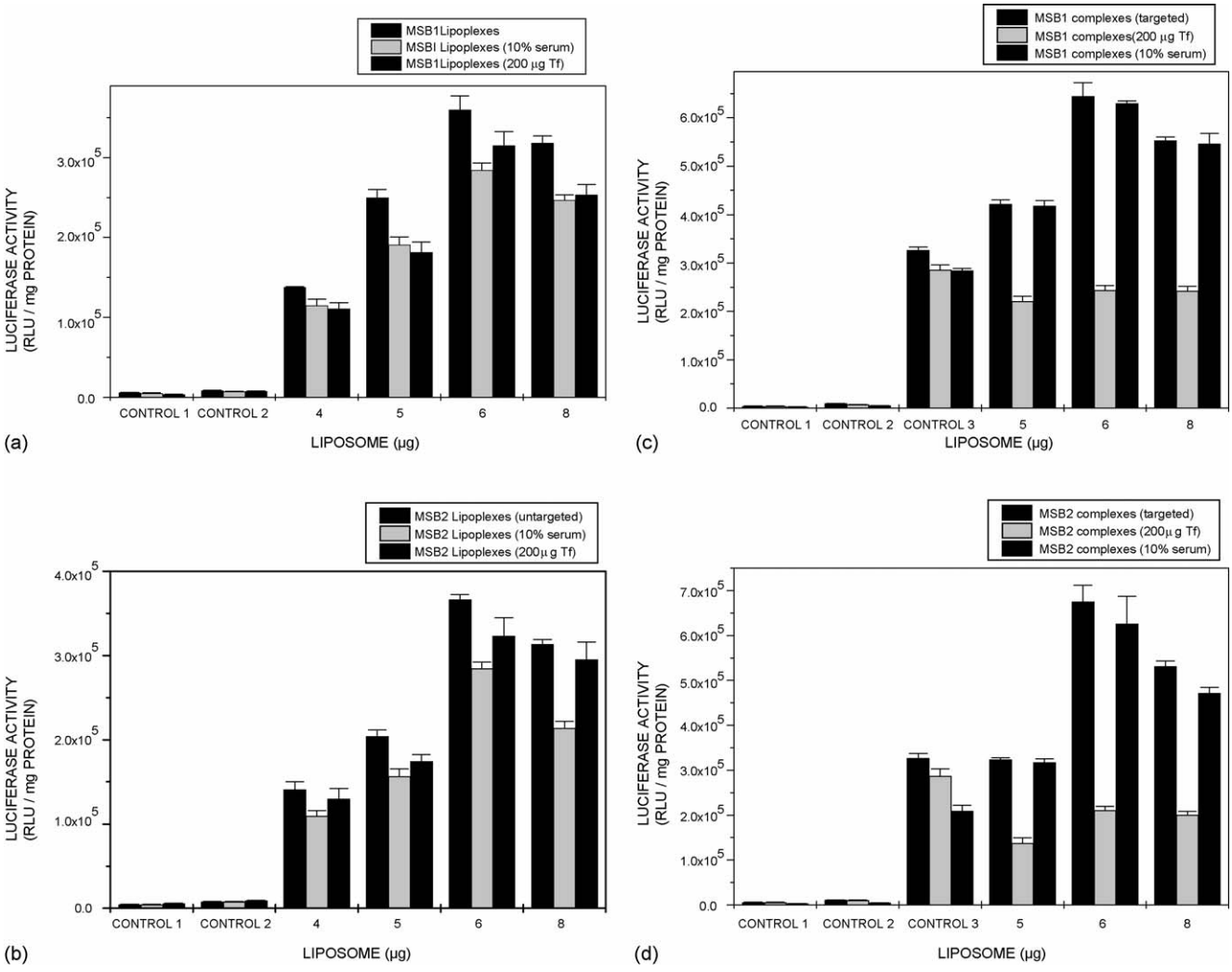

Fig. 8. Transfection studies. (a) and (b) Lipoplexes (with and without incubation in 10% serum and in the presence of 200 µg free transferrin) in HeLa cells in vitro. Liposome amount was varied (0, 4, 5, 6, 8 µg) while the DNA was kept constant (1 µg) in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposomes. (c) and (d) Transfection studies of streptavidin(bio^3-transferrin):liposome:pGL3 DNA complexes (with and without incubation in 10% serum and in the presence of 200 µg free transferrin) in HeLa cells in vitro. Liposome amount was varied (0, 5, 6, 8 µg) while the DNA (1 µg) and streptavidin(bio^3-transferrin) (10 µg) were kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells, control 2 contains naked DNA and no liposomes, and control 3 contains liposome:DNA complexes (6:1, w/w) in the absence of streptavidin(bio^3-transferrin). Data are presented as mean ± S.D. (n = 4).

Düzgüneş, 2000) where transferrin:DOTAP:DOPE lipoplexes with a charge ratio of 1:1 in the presence of 20% FBS exhibited transfection levels in HeLa cells approximately three times higher than levels achieved by plain lipoplexes without serum, while at a charge ratio of 4:1 in the absence of serum, transferrin lipoplexes were approximately four times as effective as plain lipoplexes. In a separate study (Joo and Kim, 2002) lipoplexes with a charge ratio of 2:1 to which transferrin had been covalently conjugated showed transfection levels in HeLa cells up to five times those achieved by unconjugated control liposomes. In the present study the partial uptake of complexes by receptor-mediated endocytosis was demonstrated by transfection experiments with targeted complexes in the presence of excess (200 μg) free iron loaded transferrin, where the luciferase activity was reduced by 60–70% (+/− charge ratio of 1.4:1) to levels achieved with untargeted lipoplexes (Fig. 8a and b). However, under the same conditions and at the same charge ratio untargeted lipoplexes exhibited only a 10% reduction in transfection activity. This strongly supports the notion that the increase in transfection levels observed with ternary complexes was due to the presence of the transferrin ligand and the process of receptor-mediated endocytosis. Residual transfection levels obtained in this competition assay may be due to non-receptor-mediated lipofection processes. Results obtained for ternary complexes in the presence of 10% FBS did not vary significantly from the optimal transfection activity noted for complexes in the absence of serum. Hence, these ternary complexes appear to be quite stable in medium containing 10% serum. This finding is in contrast with corresponding experiments using untargeted lipoplexes where transfection activities were reduced by 20–25% at the optimal liposome:DNA ratio of 6:1 (w/w) (Fig. 8a–d). It has been suggested that coating of lipoplexes with neutral or negatively charged proteins (e.g. transferrin) may constitute a promising strategy to modulate their colloidal stability and transfection efficiency in the presence of serum (Pedroso de Lima et al., 2001). The use of serum-free medium was found to decrease the binding of diferric transferrin to the cell surface by 30–60% whereas addition of fetal bovine serum to cells resulted in a rapid (2–3 min) concentration dependent increase in binding activity (Ward and Kaplan, 1986). Transferrin–lipoplexes are said to be superior to plain lipoplexes in transfecting HeLa cells in the presence of high concentrations of serum (Tros de Ilarduya et al., 2002). The inhibitory effect of serum seems to be more evident at lower charge (+/−) ratios of the transferrin complexes, while this inhibitory effect is overcome at higher charge ratios (+/−). Furthermore, it was reported that prolonged incubation of transferring–liposome complexes prior to addition to the cells renders them serum resistant and that maturation of the complex is an essential element of serum-resistance. The molecular basis of serum resistance and the enhancement by serum is not completely understood. It is possible that the interaction of transferrin–lipoplexes with the cell surface receptors facilitates uptake by the host cells, while plain lipoplexes are coated with serum proteins and hence inhibited from interacting electrostatically with the cell surface. The presence of transferrin on the lipoplexes could also hinder the complexation of serum proteins with the cationic lipids (Düzgüneş et al., 2003).

It is interesting to note that MSB1 lipoplexes and ternary complexes achieved transfection activities very similar to those attained by their MSB2 counterparts. The spacer length of the biotinylated liposomes seems to have little impact on the transfection activity of the two liposomes with MSB2 liposomes being slightly better in transfecting cells by only about 5% at the optimal charge ratio. Transfection also increased with increased liposome:DNA charge ratio until the same optimal transfection ratio was reached for each complex (6:1, w/w). On further increase of the +/− charge ratio transfection activity was found to decline.

4. Conclusion

We have demonstrated in these studies the first application of the streptavidin–biotin interaction in the assembly of targeted lipoplexes from biotinylated transferrin, DNA and biotinylated cationic liposomes. This is a departure from most other liposome-based transferrin-containing assemblies in which the cohesive forces which dock the transferrin component are electrostatic in nature. This assembly has been used to introduce the pGL3 plasmid carrying the luciferase gene into HeLa cells in vitro largely via the transferrin receptor which is abundantly expressed on these human cervical carcinoma cells. This stable, targeted DNA delivery complex displays undiminished efficiency in the presence of 10% foetal bovine serum and its further development therefore, seems warranted. Although the streptavidin component has been reported to exhibit spurious binding to integrins and related cell surface molecules (Das et al., 2002), it is possible that in the assemblies studied here, the streptavidin component may be partially concealed and the implicated Arg-Tyr-Asp (RYD) sequence may be sufficiently adumbrated.

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