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International Journal of Pharmaceutics 208 (2000) 41–48

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

DNA transfection and transfected cell viability using amphipathic asymmetric dendrimers

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Received 10 May 2000; received in revised form 31 July 2000; accepted 1 August 2000

Abstract

Amphipathic asymmetric dendrimers have been investigated for use in delivery of genes into cells, with the objective of optimising transfection efficiency and maintaining cell viability. We have synthesised amphipathic asymmetric dendrimers by solid phase methods. The ability of two of these to transfect BHK cells in culture with β-galactosidase gene was determined by X-gal staining. Cell viability was measured by the MTT assay for BHK cells, and by spectroscopy for lysis of erythrocytes. Interactions between dendrimer and DNA were investigated by agarose gel electrophoresis. BHK cells were optimally transfected at 5:1 +/− charge ratio yielding 20% cells receiving at least one copy of the plasmid. Cell viability decreased when the dendrimer to DNA ratio exceeded 5:1. Raising the pH significantly affected the electrophoretic mobility of complexes of dendrimer and DNA. We conclude that amphipathic asymmetric dendrimers enable efficient plasmid DNA uptake into BHK cells. Cell viability is maintained at high concentrations of dendrimer when complexed with DNA at a 5:1 +/− charge ratio. Efficiency of transfection and cell viability suggest the system may be suitable for gene delivery in vivo. © 2000 Published by Elsevier Science B.V.

Keywords: Gene delivery; Gene expression; Transfection; Dendrimers; Non-viral vectors

Abbreviations: DOTMA, 2,3-dioleyloxypropyl-1-trimethyl ammonium chloride; DOSPA, 2,3-dioleyloxy-*N*-[2(Spermine-carboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoracetate; DOGS, dioctadecylamidoglycylspermime; DOPE, dioleoyl phosphatidylethanolamine; DODAC, dioleyldimethylammonium chloride; PAMAM, polyamidoamine; G9-EDA, 9th generation PAMAN with ethylenediamine core.

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0378-5173/00/$ - see front matter © 2000 Published by Elsevier Science B.V.

PII: S0378-5173(00)00534-2

1. Introduction

Liposomes comprised of linear cationic lipids such as DOTMA, DOSPA, or DOGS are commonly used at a 5:1 +/− charge ratio to deliver plasmid DNA to permissive mammalian cells in culture with an efficiency below 10%. Commercially available transfection agent such as Lipofectin®, Lipofectamine® and Transfectam® use these lipids and often include a fusogenic co-lipid such as DOPE which can significantly enhance the efficiency of DNA delivery to the 10% level. Incorporation of a viral protein such as haemagglutinin into a liposome results in a so-called virosome. Transfection of 80% BHK cells with plasmid DNA has been achieved with virosomes containing the cationic lipid DODAC and the co-lipid DOPE at a more unusual 1:5 +/− charge ratio. Binding of the protein component specifically to the target cell surface probably obviates the need for excess positive charge (Schoen et al., 1999). Residual amounts of solvents or a non-ionic detergent may remain in the preparation of liposomes, for example $C_{12}E_8$ was used by Schoen et al., (1999).

In contrast to cationic lipids, spherical cationic dendrimers such as Starburst® (PAMAM) polymers and activated dendrimers (fractured PAMAM dendrimers) such as Superfect® do not self-associate, but like cationic lipids exhibit optimal transfection efficiency at a 5:1 +/− charge ratio. PAMAM polymers (Kukowska-Latallo et al., 1996) are water soluble but benefit from added components such as DEAE-dextran to assist endosome rupture and maximal release of DNA into the cytoplasm for effective gene expression (Tang et al., 1996). Transfection efficiency of some cell types using a G9-EDA PAMAM has recently been enhanced using the non-ionic detergent tyloxapol (a polymer of 4-(1,1,3,3-tetramethybutyl) phenol with ethylene oxide and formaldehyde) present in the synthetic lung surfactant Exosurf tail or root (Kukowska-Latallo et al., 1999). Activated cationic dendrimers, however, can transfect over 50% permissive mammalian cells in culture without co-lipids or other added components, and in particular Superfect has been used to successfully transfect corneal endothelium in ex vivo culture (Huddle et al., 1999).

We have constructed (Sakthivel et al., 1998) water soluble amphipathic molecules in the form of a branched polypeptide comprised of a dendrimeric polycation (polylysine or polyornithine) head or canopy and a hydrophobic tail or root (usually three α-amino myristic acid (C_{14}) residues) (Fig. 1). These molecules lack the symmetrical branching and high surface charge density which leads to the spherical nature of PAMAM dendrimers, but exhibit asymmetric branching due to the lysine or ornithine and have a lower surface charge density. Importantly, they are produced using a controlled synthesis yielding compounds with similarities to activated dendrimers and they avoid the chemical variability due to activation through partial hydrolysis and subsequent fractionation. These molecules, termed amphipathic asymmetric dendrimers, have been used to effectively transfect COS7 cells (SV40 transformed monkey kidney fibroblast cells) with an optimum charge ratio +/− of 5:1 (Toth et al., 1999). In the present study we demonstrate transfection of BHK-21 clone 13 cells (hamster kidney epithelial cells) with two of these compounds (see Fig. 1) in a complex with plasmid DNA, i.e. $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide and $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide with pSVβgal, at an optimum charge ratio +/− of 5:1, and show that BHK cells exhibit greater survival in the presence of the dendrimer-DNA complex than when dendrimer is applied alone.

2. Materials and methods

2.1. Dendrimers

The dendrimers were synthesised as described (Sakthivel et al., 1998), and a 1 mg/ml stock solution made in sterile distilled water. Working models of dendrimers $(\alpha/\varepsilon Lys)_7(\alpha AMA)_3$amide and $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide were generated using QUANTA96 (MSI) and energy minimised using CHARMm (MSI). The nomenclature α/εLys indicates strict use of both α and ε amino groups of the amino acid lysine when forming

peptide bonds in the branched polypeptide, and αAMA indicates use of the α amino group of amino-myristic acid when forming a peptide bond (see Fig. 1).

2.2. Plasmid DNA

The reporter plasmid pSVβgal was used to monitor gene expression following transfection. The plasmid (7.8 kb) contains the *Escherichia coli* β-galactosidase gene under the control of the Rous Sarcoma Virus long terminal enhancer/promoter to enable expression in mammalian cells. Plasmid DNA was propagated in *E. coli* DH5α, isolated by alkaline lysis, and purified twice by CsCl gradient centrifugation (Sambrook et al., 1989). In preliminary experiments, alternative plasmid purification methods using commercial kits gave similar results. DNA concentrations were determined by measuring the absorbance at

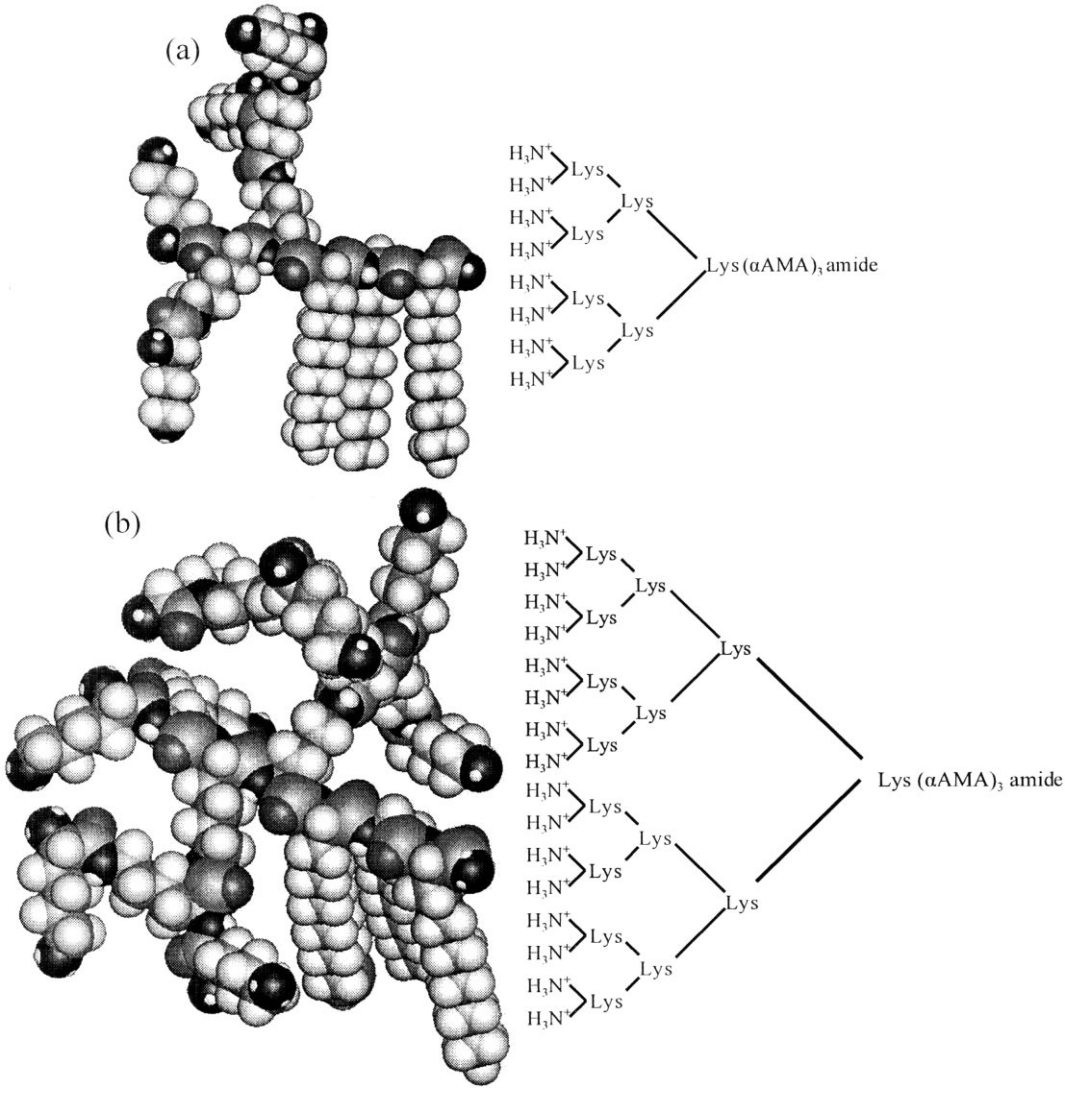

Fig. 1. Model of amphipathic asymmetric dendrimer structure. Black spheres are nitrogen atoms, terminal nitrogens are positively charged at neutral pH (a) $(\alpha/\varepsilon Lys)_7(\alpha AMA)_3$amide and (b) $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide.

260 nm and a 1 mg/ml stock solution was made in sterile distilled water. Purity was confirmed by agarose gel electrophoresis.

2.3. Dendrimer/DNA complex formation

To manufacture complexes with the required molar charge ratio, plasmid DNA (1 mg/ml) was added to dendrimer (1 mg/ml) and diluted with sterile distilled water. Where appropriate, 10 × concentrated phosphate buffered saline (PBS) was subsequently diluted into the sample. Consistency in the manufacturing process was expected to generate a consistent complex population, and although the size distribution has not been measured, zeta potentials have been previously characterised (Toth et al., 1999).

2.4. Agarose gel electrophoresis

To study the effect of pH on complex stability, different molar charge ratios of dendrimer/DNA were prepared and loaded onto a 1% agarose gel in 50 mM Tris–Acetate, 1 mM EDTA, 0.1 M NaCl buffer. No dye was present in the loading buffer (30% sucrose). Remixing anode and cathode buffers every 15 min maintained the pH between 7 and 8, but without remixing a gradient from pH 4 to 11 was achieved across the gel.

2.5. Haemoglobin release assay

Rat blood cells were obtained by centrifugation of complete blood. A 2% suspension (packed cell volume) of cells was made in phosphate saline buffer. For each dendrimer concentration, 100 μl of cell suspension was incubated with 100 μl of either dendrimer alone or dendrimer/pSVβgal complex in triplicate (96-well plate format) for 1 h at 37°C. The final concentration of pSVβgal DNA was 12.5 ng/μl. The lysate supernatant was recovered by centrifugation and the absorbance measured at 540 nm.

2.6. Cell culture

BHK-21 cells were maintained in GMEM (Glasgow's modified minimum essential media) supplemented with 10% heat activated foetal calf serum, 2 mM L-glutamine, 5% Tryptose phosphate broth, 50 U/ml penicillin, and 50 mg/ml streptomycin) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Confluent cells were subcultured twice weekly. All media and supplements were obtained from GIBCO BRL, Paisley, UK.

2.7. BHK cell survival assay

Dendrimer, DNA, and dendrimer-DNA complexes were diluted in 140 μl of phosphate buffered saline. 140 μl of serum free GMEM was added and incubated with BHK cells in triplicate at 50–60% confluence at 37°C and 5% CO_2 for 2 h using 24-well plates. The final concentration of pSVβgal DNA was 28 ng/μl. The mixture was removed and replaced with 400 μl of serum containing media. After 24 h, the cell survival was estimated by addition of 80 μl of 5 mg/ml solution of MTT. Resulting MTT-formazan was dissolved in 400 μl of isopropanol and the absorbance of MTT-formazan measured at 595 nm.

2.8. Cell transfection assay

A series of complexes of pSVβgal/$(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide were made so that the molar charge ratio +/− varied from 2.5:1–10:1 at different concentrations of $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide. The maximum concentration of compound used was 140 ng/μl. The complexes were diluted in 35 μl of phosphate saline buffer. A further 35 μl of serum free GMEM was then added and the mixture incubated on BHK cells in triplicate at about 50–60% of confluence in a 96-well plate for 2 h at 37°C and 5% CO_2. The complexes were then removed and replaced with 100 μl of serum containing media. After a further 24 h, the media was removed and the cells assayed for β-galactosidase activity. 50 μl chromogenic substrate X-gal (2 mg/ml) was added and incubated for 1 h at 37°C. Transfected cells were blue and the percentage transfection was estimated under a microscope using a graticuled field of view.

3. Results and discussion

3.1. Dendrimer structure

We have previously reported a series of dendrimeric compounds used as non-viral vectors for in vitro gene delivery (Toth et al., 1999). These were prepared by solid phase synthesis, cleaved from the support using HF, and characterised by mass spectrometry (Sakthivel et al., 1998). The structures of the amphipathic asymmetric dendrimers, $(\alpha/\varepsilon Lys)_7(\alpha AMA)_3$amide and $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide, used in this study are shown in Fig. 1. The structures shown in the figure are chemically definitive but have sufficient flexibility to adopt many conformations of equivalent energy in water. Energy minimisation has been used simply to remove bad contacts, bond angles and bond lengths. The terminal amines of the dendrimer will be protonated at physiological pH and, although hydrated, will tend towards a conformation with a charge distribution evenly dispersed. However, the conformation can readily adapt by rotation around the many lysine methylene–methylene bonds to juxtapose several positive charges on the surface of the dendrimer for interaction with the negative charges on the less flexible DNA (or cell surface proteins) and thereby generating a binding affinity. The high generation number PAMAM polymers capable of transfecting cells with DNA have a high surface density of terminal amines and are therefore sterically constrained. An increase in flexibility of the surface charges in activated (or fractured) PAMAM dendrimers results from some 17 to 33% of the branches being pruned by random hydrolysis, and is thought to explain the enhanced transfection rates observed in that case (Tang et al., 1996). The amphipathic asymmetric dendrimers we have described are inherently flexible.

3.2. Dendrimer-DNA complex formation

In these studies the DNA was added to the dendrimer in a relatively concentrated aqueous solution to form the complex, and then diluted into the appropriate medium necessary for growth of cells in culture. This approach avoids the complication of culture medium components interfering with complex formation, but limits the maximum final concentrations at which the complex can be used. High molecular weight aggregates of dendrimer-DNA complexes have been observed using agarose gel electrophoresis in which the complex cannot migrate from the loading well. The same lack of migration is observed whether the complex is formed in the presence of minimal cell culture media, e.g. OptiMEM, or with other transfection agents such as cationic lipids or PAMAM dendrimers. The effect seems to occur virtually immediately after the dendrimer and DNA are mixed, within the 2–3 min it takes to load an agarose gel and start electrophoretic separation. However, we observed that the high molecular weight complex appears to dissociate equally quickly if the pH in the agarose gel is raised to pH 11. We performed agarose gel electrophoresis in standard TAE buffer but with 0.1 M NaCl and, using a mini-gel apparatus, the pH in the anode and cathode reservoirs rapidly polarised to create a pH gradient across the gel between pH 4 and 11. As a control, the reservoirs

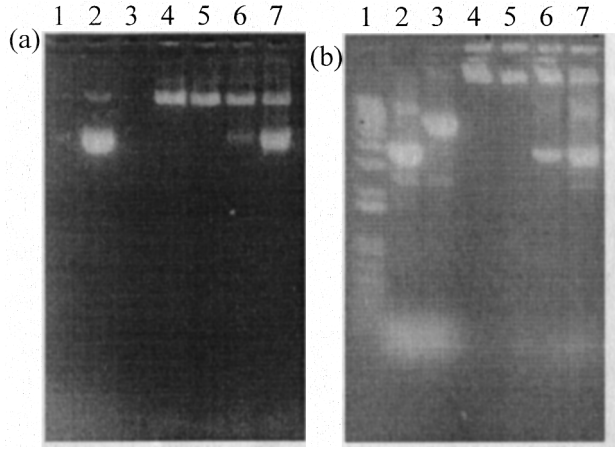

Fig. 2. pH dependence of complex stability. Identical samples of markers and plasmid pSVβgal (100 ng) were loaded onto both gels. (a) Agarose gel pH gradient 4–11 (b) Agarose gel pH 7–8. Lane numbers are as follows: lane 1, markers; lane 2, stock plasmid; lane 3, *Hin*dIII cut plasmid; lanes 4–7 are DNA/$(\alpha/\varepsilon Lys)_7(\alpha AMA)_3$amide complexes at 10:1, 5:1, 1:5, 1:10 +/− charge ratio, respectively.

were mixed at 15 min intervals to maintain pH 7–8 in the reservoirs. To achieve separation of DNA in the presence of 0.1 M NaCl the electrophoresis was carried out for 3 h instead of the normal 20 min. Bromophenol blue was not used in the loading buffer since the dye was suspected of binding to the dendrimer complexes and absorbing fluorescent light during DNA visualisation (data not shown). Fig. 2 shows that the dendrimer-DNA remains in the well at pH 8 (Fig. 2b), but migrates into the gel at pH 11 (Fig. 2a). Interestingly at pH 11 at 10:1 and 5:1 +/− charge ratios the DNA migrates to the position observed for linear DNA rather than supercoiled DNA, whereas at 1:5 and 1:10 ratios there is less intensity at the 'linear position' and more has migrated to the 'supercoiled position'. DNA at

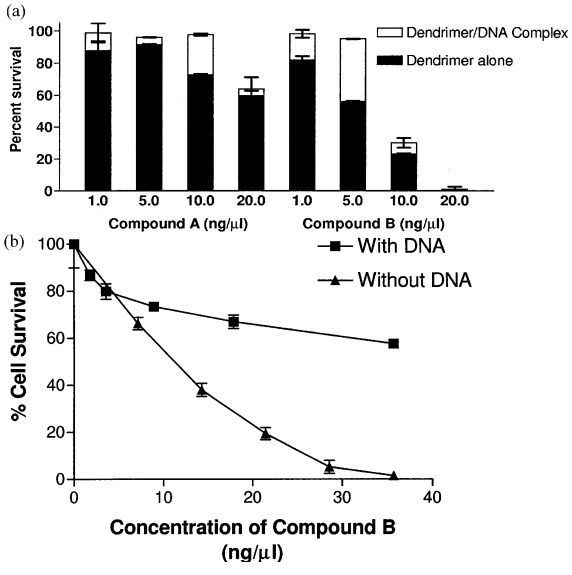

Fig. 3. (a) Red blood cell viability. Mean percent survival (triplicate) in the presence of $(\alpha/\varepsilon Lys)_7(\alpha AMA)_3$amide (A) and $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide (B). Values were scaled between 100% (addition of PBS) and 0% (addition of Triton X-100) by assaying haemoglobin release. The final concentration of pSVβgal DNA was 12.5 ng/μl. Error bars show standard deviation from mean. (b) BHK cell viability. Mean percent survival (triplicate) in the presence of $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide (compound B) Values were scaled between 100% (addition of PBS) and 0% (addition of Triton X-100) by MTT assay. The final concentration of pSVβgal DNA was 28 ng/l. Error bars show standard deviation (S.D.) from mean.

the 'supercoiled position' which occured at either pH may simply indicate no bound dendrimer, or that a low molar ratio has minimal effect on molecular weight and hence has a minimal effect on migration. The intensity of the band at the 'linear position' is however dendrimer concentration dependent and pH dependent indicating an effect of the dendrimer on the molecular weight or conformation of the DNA. It is not clear whether at pH 11 it is a small amount of bound dendrimer causing relaxation of the supercoiling, or a large amount which, although no longer able to cross-link and form aggregates, is able to bind and increase the molecular weight to be coincident with the linear DNA band. It cannot be linear DNA since the strands would separate and would not intercalate ethidium bromide as in the case of markers in lane 1 and *Hin*dIII cut DNA in lane 3. However, the effect is rapid, as expected for an ionic interaction between cationic dendrimer and anionic DNA.

3.3. Dendrimer-DNA complex less toxic than dendrimer alone

Fig. 3a shows the effect of DNA-dendrimer complex on rat erythrocyte integrity. It was found that exposure to dendrimer alone at ten-fold higher concentrations than that previously used with COS7 cells (Toth et al., 1999) leads to release of haemoglobin from rat erythrocytes. When the dendrimers were added as complexes with DNA, this concentration was better tolerated. More than 30% erythrocytes were lysed with ≥ 50 ng/μl $(\alpha/\varepsilon Lys)_7(\alpha AMA)_3$amide in the absence of DNA, whereas less than 5% were lysed with ≥ 50 ng/μl in the presence of DNA. The same was true at the 25 ng/μl level for $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide. This suggests that lysis is correlated with a critical level of free amino groups since $(\alpha/\varepsilon Lys)_7(\alpha AMA)_3$ amide has eight free amino groups and $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide has 16 free amino groups.

We have also assayed for growth inhibition by the dendrimers. The ability of BHK cells to divide in culture may be more sensitive to dendrimers than suggested by the erythrocyte assay which only measures lytic activity. A sub-confluent

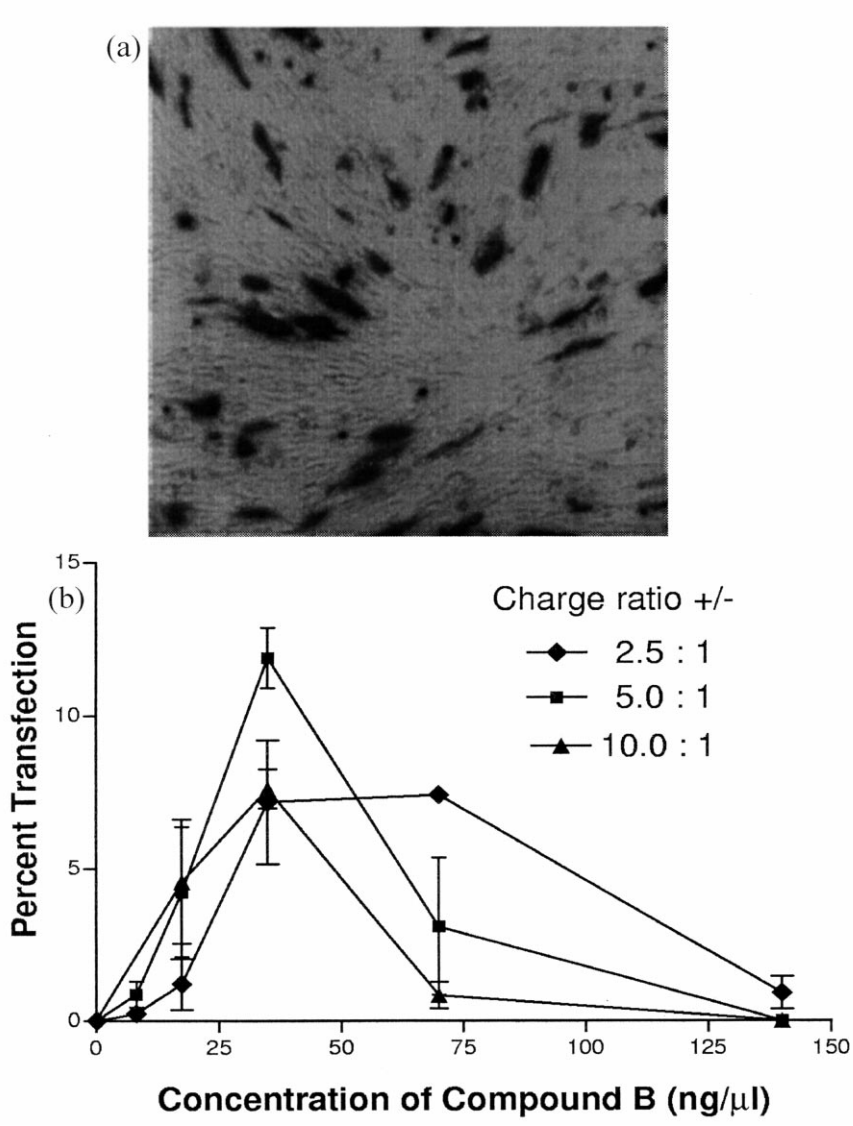

Fig. 4. (a) Transfection efficiency: BHK cells transfected with $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide/ pSVβgal stained blue by incubation with β-galactosidase substrate X-gal. (b) Transfection efficiency vs concentration of $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide at three molar charge ratios. Mean percentage transfection (triplicate) measured as in (a) but with 2.5:1, 5:1, 10:1 $+/-$ molar charge ratio. Error bars show S.D. from mean.

monolayer of cultured cells were exposed to the dendrimer for 2 h, then maintained in culture for a further 24 h before the surviving cells were assessed (Fig. 3b). 30% failed to survive with ≥ 5 ng/μl $(\alpha/\varepsilon Lys)_{15}(\alpha AMA)_3$amide alone and virtually none survived at ≥ 35 ng/μl, whereas in the presence of DNA more than 60% survived at ≤ 35 ng/μl. This shows an order of magnitude greater sensitivity of the BHK cells growing in culture to dendrimer applied in the absence of DNA when compared with erythrocyte lysis. Importantly for transfections, the sensitivity to dendrimer is significantly overcome when an appropriate concentration of DNA forms a com-

plex with the dendrimer. The sensitivity of cells growing in culture to DNA-dendrimer complex is closer to the erythrocyte lysis level.

3.4. Transfection and β-galactosidase expression in BHK cells

We demonstrated earlier that these compounds are capable of delivering plasmid DNA to some cell lines by assaying luciferase activity from lysates of transfected COS cells (Toth et al., 1999). We have now transfected BHK cells with a larger plasmid, observing that β-galactosidase activity was present in 10% of cells available for transfection (Fig. 4a). Optimal transfection of BHK cells was observed at 35 and 14 ng/μl DNA at a 5:1 +/− molar charge ratio (Fig. 4b). In comparison, doubling the dendrimer concentration results in an optimum transfection at the lower 2.5:1 +/− charge ratio, consistent with protection by a higher proportion of DNA (56 ng/μl). However, doubling the dendrimer concentration at the 5:1 +/− molar charge ratio results in a much reduced transfection level, consistent with toxicity of excess dendrimer. Addition of DEAE-dextran or chloroquine, at concentrations used to enhance transfection with cationic liposomes, had no effect on the transfection efficiency of these compounds indicating that these compounds are capable of moving out of the endosome without assistance, and so are more akin to activated dendrimers than to liposomes.

4. Conclusions

Cationic amphipathic dendrimers based on lysine with amide linked lipids produced by solid-phase synthesis were investigated as gene delivery vectors. BHK-21 cells were optimally transfected with complexes of dendrimers bearing 8 or 16 terminal amines and plasmid DNA at a molar charge ratio +/− of 5:1. In this system, BHK cells exhibited greater survival in the presence of the dendrimer-DNA complex compared with dendrimer alone. There was no enhancement of transfection with added agents such as DEAE-dextran or chloroquine. The interaction between dendrimer and DNA in aqueous solution was an ionic process, which could be rapidly reversed at pH 11. A monomer model has highlighted the flexibility of these amphipathic asymmetric dendrimers. The compounds have flexibility in the dendrimeric head similar to activated dendrimers, but with a sufficiently exposed hydrophobic tail for interactions with target cell membrane and potential to form an oligomer.

Acknowledgements

D. Shah was supported by a School of Pharmacy Millennium Studentship, T. Sakthivel is a Maplethorpe Postdoctoral Fellow, University of London.

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