International Journal of Pharmaceutics 378 (2009) 201–210

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Effect of acyl chain length on transfection efficiency and toxicity of polyethylenimine

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ARTICLE INFO

Article history:
Received 22 March 2009
Received in revised form 18 May 2009
Accepted 25 May 2009
Available online 6 June 2009

Keywords:
Polyethylenimine
Buffering capacity
Nonviral vectors
Haemolysis
Transfection
Biocompatibility

ABSTRACT

Polyethylenimine (PEI) is an efficient nonviral gene delivery vector because of its high buffering capacity and DNA condensation ability. In our study, the amino groups on the polymeric backbone were acylated using acetic or propionic anhydride to alter the protonation behaviour and the hydrophilic/hydrophobic balance of the polymer. The concentration of acylated primary amines was determined using trinitrobenzene sulphonic acid assay. Results showed that our modified polymers had lower buffering capacities in solutions compared to PEI. The polymers were complexed with plasmid encoding enhanced green fluorescent protein at three different ratios (1:1, 1:2 and 1:10 w/w DNA to polymer) to form polyplexes and their toxicities and transfection efficiencies were evaluated in HEK 293 cells. Acylation reduced the number of primary amines on the polymer and the surface charge, improving haemocompatibility and reducing cytotoxicity. The reduction in the concentration of amino groups helped to optimise DNA compaction and facilitated polyplex dissociation in the cell, which increased transfection efficiency of the modified polymers compared to the parent polymer. Polymers with buffering capacities greater than 50% and less than 80% relative to PEI, showed higher transfection efficiencies than PEI. The propionic anhydride modified polymers had appropriate interactions with DNA which provided both DNA compaction and polyplex dissociation. These systems interacted better with the cell membrane because of their slightly higher lipophilicity and formed polyplexes which were less cytotoxic than polyplexes of acetic anhydride modified polymers. Among the vectors tested, 1:0.3 mol/mol PEI:propionic anhydride in a 1:2 w/w DNA:polymer composition provided the best transfection system with improved transfection efficiency and reduced cytotoxicity.

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1. Introduction

Gene therapy offers an exciting and novel therapeutic strategy for both genetic and acquired diseases (Brooks, 2002). DNA or RNA can be used as drugs to replace defective genes with functionally correct sequences to restore expression or to regulate unwanted gene expression (Brooks, 2002). Successful therapy requires efficient gene transfer and target specificity without inducing a significant immune response or reducing expression or regulation of the therapeutic gene (Brewster et al., 2006; Tiera et al., 2006). Efficient gene transfer requires a suitable carrier that can protect the therapeutic DNA from degradation within the cell before it reaches the nucleus, which has prompted extensive research into developing competent delivery vectors, which also possess low cellular toxicity (Brewster et al., 2006; Tiera et al., 2006). The death of a patient from virus-mediated gene therapy in 1999 and the development of cancer in children treated for severe combined immunodeficiency in 2002 moderated the early enthusiasm for using viral gene delivery vectors (Godecke, 2006). Although the first commercialised gene therapy formulation, Gendicine, uses a recombinant adenovirus, the safety of the viral vector remains controversial (Xin, 2006). Whilst viral vectors raise issues of safety and scalability, non-viral vectors present issues concerning efficiency of transfection, biocompatibility, cytotoxicity and target specificity (Tiera et al., 2006). Attempts are now being made to combine the efficacy of viruses with the safety profile of polymers (Fisher et al., 2007); however, despite numerous efforts, ideal gene transfer carriers remain elusive.

Of the most extensively studied polymers for gene delivery, polyethylenimine (PEI) is of considerable interest, because it shows high transfection efficiency in differentiated as well as non-differentiated cells (Florea et al., 2002). PEI is a commercially available cationic polyamine first introduced as a gene carrier by Boussif et al. (Boussif et al., 1995). It is readily taken up by cells through endocytosis and escapes endosomal degradation through the proton-sponge effect because of its cationic nature (Boussif et al., 1995; Sonawane et al., 2003; Akinc et al., 2005). Many factors such as molecular weight, degree of branching, ionic strength of

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doi:10.1016/j.ijpharm.2009.05.052

the solution, zeta potential, particle size, cationic charge density, molecular structure, sequence and conformational flexibility have all been shown to affect transfection efficiency and cytotoxicity (Choksakulnimitr et al., 1995; Fischer et al., 1999; Godbey et al., 1999a; Wightman et al., 2001; Fischer et al., 2003; Lv et al., 2006). Since the amino groups on the polymeric backbone are responsible for condensing DNA and for the proton-sponge effect, modifications of the amino groups have been explored to further improve PEI's transfection efficiency and reduce cytotoxicity.

As previously shown (Forrest et al., 2004; Gabrielson and Pack, 2006), acylating the amino groups of PEI using acetic anhydride decreased the buffering capacity of the polymer and weakened electrostatic interactions with DNA, which improved transfection efficiency by facilitating polyplex unpacking within the cell. However, the alkylation of 25 kDa PEI's primary amino groups by dodecylation reduced transfection efficiency and cytotoxicity in COS-7 cells (Thomas and Klibanov, 2002). Although the increase in hydrophobicity might be expected to improve cellular uptake by enhancing polyplex interaction with the cell membrane, clearly there is a need to maintain the hydrophobic/hydrophilic balance of the nonviral vector. Nimesh et al. (Nimesh et al., 2007) studied transfection efficiency as a function of acyl chain length using nanoparticles formed by acylation of 750 kDa PEI followed by crosslinking with PEG; transfection efficiency improved 5–12 fold compared with native PEI in COS-1 cell line, but it is not clear if the improvement was caused by acylation or PEGylation.

In this study, we have systematically modified branched 25 kDa PEI and extended previously published work by comparing acetic anhydride and propionic anhydride as acylating agents to observe the effects of acyl groups on the pendant chains of the polymeric backbone. DNA condensation, buffering capacity, transfection efficiency and toxicity are investigated as a function of the acyl chain length. We have shown that subtle changes in hydrophobicity considerably alter the buffering capacity and the condensation ability of the polymer which consequently affects transfection efficiency and toxicity of the nonviral vectors. Our results show that PEI acylated with propionic anhydride is more efficient than PEI acylated with acetic anhydride in transfecting cells.

2. Materials and methods

2.1. Materials

Branched polyethylenimine (MW 25 kDa) was obtained from Sigma–Aldrich, UK. Acetic anhydride, methanol (HPLC grade), sodium hydroxide, hydrochloric acid, dimethyl sulphoxide and borate buffer (pH 9.2) were from Fisher Scientific, UK and propionic anhydride was obtained from Acros Organics, UK. Picrylsulphonic acid was obtained from Sigma–Aldrich, UK. Dialysis membranes (molecular weight cutoff 3500 Da) were from Medicell International Ltd, UK. Deuterated water was from GOSS Scientific Instruments Ltd, UK. Spectroscopic grade potassium bromide was obtained from Graseby Specac Ltd, UK. All materials were used as received. All reagents were lab reagent grade unless otherwise stated and deionised water was used for all experiments.

XL-1 Blue cells were obtained from Stratagene, California, USA. The mammalian expression plasmid, pIRES2-EGFP, was purchased from Clontech, USA. Human embryonic kidney cells (HEK 293) cells were from Qbiogene Inc, California, USA. Human blood cells were kindly supplied by Dr. David Leake, University of Reading. Luria Broth (LB) agar, LB broth base, agarose (electrophoresis grade) and ethidium bromide (electrophoresis grade) were purchased from Invitrogen, UK. Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS) and trypsin-EDTA solution were purchased from Gibco, UK. Genelute HP plasmid megaprep kit was obtained from Sigma, UK. MTT reagent thiazolyl blue tetrazolium bromide, phosphate buffered saline (PBS) and Triton X-100 were obtained from Sigma–Aldrich, UK.

2.2. Synthesis of acylated PEI

Synthesis of acylated PEI was performed as published previously, with minor modifications (Forrest et al., 2004). PEI (1 g = 23 unit-base mmol) was dissolved in 10 mL of methanol. Acetic anhydride or propionic anhydride was added in varying quantities to obtain feed ratios of 1:0.1, 1:0.3, 1:0.5, 1:1 and 1:2 mol/mol PEI to acylating agent. Acylation was carried out at 60 °C with moderate shaking for 4.5 h. The reactions were quenched with 1 mL of distilled water and methanol was removed using a rotary evaporator. The resulting solutions were dialysed (molecular weight cutoff 3500 Da) against 2 L of water (7 changes in 5 days), which provided viscous yellow solutions. These were frozen and lyophilized using an IEC Lyoprep-3000 freeze drier to obtain acylated PEIs, which were characterized by Fourier transform infrared spectroscopy using a PerkinElmer 1720-X spectrometer as the average of 16 scans from 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1}. IR (KBr) of acetic anhydride modified PEI (ACAN) υ (cm^{-1}): 3408 (secondary amide N–H stretching), 3077 (primary amide N–H stretching), 2951, 2839 (C–H stretching), 1633 (carbonyl stretching), 1568 (N–H bending). IR (KBr) of propionic anhydride modified PEI (PRAN) υ (cm^{-1}): 3411 (secondary amide N–H stretching), 3068 (primary amide N–H stretching), 2947, 2834 (C–H stretching), 1635 (carbonyl stretching), 1560 (N–H bending).

2.3. Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR for PEI was recorded on a Bruker AMX 400 spectrometer at 100 MHz with 11,500 scans and a delay of 20 s between each scan. The percentage of primary, secondary and tertiary amines in the polymer was calculated as reported previously (Von Harpe et al., 2000). Unmodified PEI and the 10 acylated PEI polymers were dissolved in D_2O (10 mg/mL) in 5 mm NMR tubes and ^1H NMR spectra were recorded on a Bruker DPX 250 MHz spectrometer at 250 MHz with 16 scans and a delay of 1 s between each scan.

2.4. Trinitrobenzene sulphonic acid (TNBS) assay

TNBS assay was used to determine the percentage of primary amines in the polymers (Habeeb, 1966). Briefly, polymer solutions were prepared at 0.01 mg/mL in borate buffer (pH 9.2). To 1 mL of each solution, 25 μL of 0.03 M aqueous TNBS was added and the solutions were incubated at room temperature for 30 min, after which the absorbance was read at 420 nm using a Jasco 530-V UV spectrophotometer. Absorbances were read in triplicate and the modification efficiency was calculated according to Eq. (1).

$$\text{Modification percentage} = \left(1 - \frac{A_{\text{mod}}}{A_{\text{unmodified}}}\right) \times 100 \quad (1)$$

where A_{mod} = absorbance of modified polymer and $A_{\text{unmodified}}$ = absorbance of unmodified polymer at 420 nm.

2.5. Buffering capacity of the modified polymers

pH titrations of the polymers were performed as described previously (Gabrielson and Pack, 2006). Briefly, 2 mg of the polymers were dissolved in 5 mL of deionised water and the pH was adjusted to 11.5 using 1 M sodium hydroxide. Aliquots (5 μL) of 1 M hydrochloric acid were added sequentially and pH was measured using a Metrohm 713 pH-meter. Buffering capacity was calculated as the volume of hydrochloric acid added to decrease the pH by 1 unit in the pH range 4.5–7.5 and is reported as mean ± SD (n = 3).

2.6. Haemolysis assay

The release of haemoglobin from red blood cells was used to evaluate the haemocompatibility of our modified polymers according to the protocol of Kan et al. (Kan et al., 2005), with minor modifications. Human blood (5 mL) was collected in heparinised tubes and homogenously dispersed with the anticoagulant. The plasma and buffy coat were removed by centrifugation at 700 × *g* for 20 min. The erythrocyte pellet obtained was washed three times with PBS (pH 7.4) by centrifuging at 1000 × *g* for 10 min at 4 °C and resuspending in the same buffer. A 3% (w/v) erythrocyte suspension in PBS was prepared, placed on ice and used immediately. Our polymer solutions were prepared at different concentrations in PBS; 80 μL of each polymer solution was added to 80 μL of the erythrocyte suspension in a 96 well microtitre plate and incubated at 37 °C for 1 h. The microtitre plate was then centrifuged at 1000 × *g* for 10 min and 100 μL of the supernatant was transferred to a new microtitre plate. The supernatant was analysed spectrophotometrically at 540 nm using a Molecular Devices Emax microplate reader. Triton X-100 and PBS were used as controls to provide 100% and 0% haemolysis, respectively. Haemolysis percentages were calculated according to Eq. (2).

$$\text{Haemolysis} = \frac{A_s - A_n}{A_p - A_n} \times 100 \quad (2)$$

where A_s is the absorbance of the cell supernatant treated with polymer sample, A_n is the absorbance of cell supernatant treated with the negative control PBS, A_p is the absorbance of cell supernatant treated with the positive control Triton X-100. All assays were performed in triplicate and data are reported as mean ± SD.

2.7. Plasmid preparation and characterization

Competent XL-1 Blue cells were transformed with pIRES2-EGFP plasmid encoding the enhanced green fluorescent protein using standard conditions and grown in LB medium containing 50 mg/mL kanamycin (Sambrook and Russell, 2001). The plasmid was purified using a commercial purification kit (Genelute maxiprep) according to the manufacturer's protocol. The purity of the plasmid was confirmed by measuring absorbance at 260 and 280 nm, A_{260}/A_{280} was 1.8. The plasmid purity was also confirmed by restriction enzyme digestion followed by agarose gel electrophoresis on a 1% agarose gel and the concentration was determined by UV spectrophotometry according to Eq. (3).

$$\text{Concentration}\,(\mu g/mL) = A_{260\,nm} \times 50 \quad (3)$$

2.8. Ethidium bromide fluorescence assay

The fluorescence intensity of the polymer complexes with DNA was evaluated using the ethidium bromide fluorescence assay (Geall and Blagbrough, 2000). Polymers were prepared at varying concentrations from 1 to 100 μg in 250 μL PBS buffer and were added to a 96-well microtitre plate. DNA (1 μg) was added to each of the wells and incubated at room temperature for 10 min to form polyplexes (complexes of polymer and DNA) at 1:100, 1:50, 1:10, 1:5 and 1:1 ratios (DNA: polymer w/w ratio). Ethidium bromide (3 μL of 0.5 mg/mL in PBS) was added to each well. The blank contained only the buffer with ethidium bromide and the positive control contained DNA with ethidium bromide. The solutions were excited at 492 nm and the emission was recorded at 620 nm using a Tecan Genios microplate reader. The fluorescence intensity values were calculated according to Eq. (4).

$$\text{Fluorescence intensity} = \frac{F_s - F_b}{F_c - F_b} \times 100 \quad (4)$$

where F_s is the fluorescence intensity of the sample, F_b is the fluorescence intensity of blank and F_c is the fluorescence intensity of the positive control. All assays were performed in triplicate and data are reported as mean ± SD.

2.9. Size and zeta-potential of polyplexes

Polyplexes at 1:10 DNA to polymer w/w ratio were prepared immediately prior to analysis. The size (hydrodynamic diameter) and zeta-potential were measured using a Malvern Instruments Nano-ZS Nanoseries Zetasizer, UK. Data reported are mean ± SD, $n = 3$.

2.10. Transmission electron microscopy

Polyplexes were formed at 1:10 DNA to polymer w/w ratio. The samples were loaded on thin carbon films supported on a 3 mm copper grid, dried and imaged (without any staining) using a Philips CM20 analytical transmission electron microscope under an acceleration voltage of 80 kV. The images were recorded photographically, which were then scanned to obtain digital images, before processing and particle sizing using a Scandium analysis package (Olympus Soft Imaging Solutions).

2.11. Cytotoxicity of polymers and polyplexes

Cytotoxicity of our polymers was assessed using the MTT assay (Mosmann, 1983). MTT reagent was dissolved in PBS buffer at 5 mg/mL and filtered before use. HEK 293 cells were plated at 10^4 cells/well and allowed to adhere to the plate. After 24 h, the medium was removed (without disturbing the cells) and replaced with varying concentrations of polymer solutions. The volume was then adjusted to 100 μL using complete medium (containing 5% serum). After incubation for 6 or 24 h, the medium containing the polymers was removed, the wells were washed with PBS and complete medium was added. The assay was performed 48 h after initial plating of the cells. MTT reagent (20 μL) was added to each well and the plates were incubated at 37 °C for 5 h. The medium was again removed and the dark blue crystals that had formed were dissolved in 100 μL of DMSO. After incubating at 37 °C for 30 min, the absorbance was read at 540 nm using a Tecan Genios microplate reader. Cell viability was calculated according to Eq. (5).

$$\text{Cell viability} = \frac{A_s}{A_c} \times 100 \quad (5)$$

where A_s is absorbance measured for cells incubated with our polymers and A_c is absorbance measured for the control untreated cells. Assays were performed in triplicate and the entire experiment was repeated three times. Concentrations were plotted against percentage cell viability for each experiment and the concentration corresponding to 50% cell viability was determined to obtain IC_{50} values. Further MTT assays were also done with polyplexes formed at 1:1, 1:2 and 1:10 w/w DNA: polymer and were compared with similar concentrations of solutions containing only polymers. For these studies, the incubation period of polymers or polyplexes with the cells was 24 h.

2.12. Transfection

Transfection of HEK 293 cells using our polyplexes was carried out according to a previously reported procedure with some modifications (Vancha et al., 2004). HEK 293 cells were cultured in DMEM medium with Glutamax, supplemented with 5% heat-inactivated FCS at 37 °C in a 5% CO_2 enriched environment. Once the cells reached about 70% confluency, they were trypsinised using 0.05% trypsin/EDTA and seeded in 12 well plates at a density of 5×10^4

cells per well. After 24 h, the medium in the wells was replaced by transfection medium containing 5% serum and the polyplexes, such that each well contained 1 μg of DNA. The polyplexes were prepared by adding 1 μg of DNA to appropriate amounts of polymer sample in PBS to obtain 1:1, 1:2 and 1:10 w/w ratios of DNA to polymer, followed by light vortexing and incubation at room temperature for 15 min. Medium containing transfection complexes was replaced with complete medium after 6 or 24 h incubation with the cells. Fluorescence images were obtained after 48 h on a Nikon Eclipse TE 200 using Lucia image processing and analysis software. The experiment was performed in triplicate. Transfection efficiency was calculated as the percentage of fluorescent cells relative to the total number of cells.

2.13. Statistical analysis

Cytotoxicity and transfection data were analysed by the unpaired two-sample *t*-test using Genstat software (version 10.2) to compare differences between means. The effect of incubation period on cytotoxicity and transfection was also analysed. Significance was defined at $P < 0.05$.

3. Results

3.1. Synthesis and characterisation of acylated polymers

Commercially available branched 25 kDa PEI was acylated using acetic anhydride or propionic anhydride to modify the primary and secondary amines on the polymeric backbone to secondary and tertiary amides, substituted with the acetyl (ACAN) or propionyl group (PRAN) (Fig. 1). The modified polymers were characterised by NMR spectroscopy. From our ^{13}C NMR studies, the percentages of primary, secondary and tertiary amines in PEI were found to be 31%, 41% and 28%, respectively and the degree of branching was 1.46. However, the ^{1}H NMR spectra were not well resolved for quantitative studies; the unsuitability of the spectra was confirmed by 2D-COSY spectra, which showed coupling interactions between the different proton signals. An alternative technique based on UV–vis spectroscopy was used to quantify the percentage of acylated amines. TNBS reacts with primary amino groups of the polymer (Johnson and Klotz, 1974) and can be used to determine the percentage of free primary amines in the polymer samples, which can consequently be used to estimate the percentage of acylated primary amines in our polymers. The results showed increasing modification percentage of the polymer with increasing molar ratios of the acylating reagent used (Table 1). As the length of the acyl substitution increased, the modification percentage decreased; this could be because of the increased steric hindrance of the longer acyl groups. The reduction in concentration of primary amines decreases the concentration of protonable nitrogens of the polymer, which might affect buffering capacity and DNA binding and hence, toxicity and transfection.

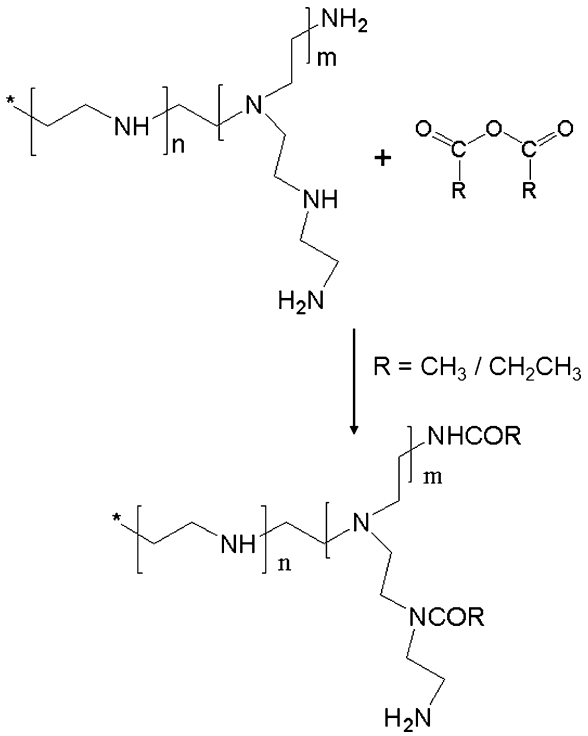

Fig. 1. Synthesis of acylated polyethylenimine using acetic or propionic anhydride.

3.2. Buffering capacity of acylated polymers

Optimal buffering capacity at endosomal pH is essential to allow endosomal escape and polyplex release into the cell cytoplasm. The buffering capacity of the polymers was calculated as the slope of the pH titration curves between pH 4.5 and 7.5. PEI showed a high buffering capacity whilst the modified polymers had lower buffering capacities. The reduction in buffering capacity depended on both the degree of acylation and on the acylating agent used (Table 1). As the degree of acylation increased, buffering capacity reduced because of the reduction of surface charge caused by the inclusion of non-ionic acyl groups in the polymeric structure. The modification percentages of 0.5 PRAN and 0.3 ACAN were almost the same, yet 0.5 PRAN showed a higher reduction in buffering capacity compared to 0.3 ACAN, which could be because of the conformational differences between the acetic anhydride and propionic anhydride modified polymers. The highly modified polymers 0.5 ACAN, 1 ACAN, 2 ACAN, 1 PRAN and 2PRAN, showed buffering capacities less than 3 μL/pH unit over the pH range 4.5–7.4 and did not transfect the cells in any of the three DNA:polymer ratios we tried. These polyplexes could be sequestered inside the endosomes and cleared rapidly, making the plasmid unavailable for transfection as confirmed by our transfection studies.

3.3. Haemolysis assay

The compatibility of any vector with blood is clearly important and indicates its suitability for introduction into the systemic circulation (Brownlie et al., 2004). PEI caused aggregation of red blood cells, while the modified polymers did not cause aggregation. PEI also showed haemolytic activity that increased with increasing concentration of the polymer; around 20% haemolysis was detected at 1 mg/mL which is broadly in agreement with the results of Brownlie et al. (Brownlie et al., 2004). However, the polymers modified using acetic anhydride or propionic anhydride were more compatible with red blood cells and haemolysis decreased with increasing acylation. While the highly modified polymers were haemocompatible at all the concentrations studied, both 0.1 ACAN and 0.1 PRAN showed increased haemolysis with increasing concentrations and at 1 mg/mL lysed 2% and 4% of red blood cells, respectively (Fig. 2); the PRAN modified polymers generally caused higher haemolysis than ACAN modified polymers.

As this test essentially measures the amount of haemoglobin released on damage of the red blood cell membrane, it can be concluded that the acylated derivatives cause less membrane damage than unmodified PEI. The high membrane damage potential of PEI could be because of the cationic charge, which improves interaction with membrane proteins and phospholipids and thus disturbs

Table 1
Percentage modification of primary amines and buffering capacity of unmodified PEI and PEI derivatives.

Sample code	Acylating agent	(PEI)/(acylating agent) ratio (mol/mol)	Modification percentage (from TNBS)	Buffering capacity[a] (μL/pH unit)
PEI	–	Unmodified PEI	0	10.38 ± 0.77
0.1ACAN	Acetic anhydride	1:0.1	37 ± 4	8.96 ± 0.77
0.3 ACAN	–//–	1:0.3	76 ± 3	5.54 ± 0.06
0.5ACAN	–//–	1:0.5	91 ± 9	2.71 ± 0.88
1ACAN	–//–	1:1	102 ± 8	1.91 ± 0.35
2ACAN	–//–	1:2	98 ± 2	2.42 ± 0.60
0.1PRAN	Propionic anhydride	1:0.1	31 ± 7	9.12 ± 1.83
0.3PRAN	–//–	1:0.3	72 ± 5	6.00 ± 0.71
0.5PRAN	–//–	1:0.5	75 ± 1	2.67 ± 0.71
1PRAN	–//–	1:1	97 ± 2	2.17 ± 0.24
2PRAN	–//–	1:2	90 ± 13	1.46 ± 0.77

[a] Values expressed as mean ± SD, $n = 3$.

membrane structure and function (Morgan et al., 1989; Fogera et al., 2006). Acylation decreases the positive charge density and the conformational flexibility of the polymer, which reduces interactions with negatively charged red blood cell membranes and so reduces haemolysis. The decrease in the concentration of primary amines of the polymers also reduces haemolysis, because primary amines are known to exert toxic effects on erythrocyte membranes, while tertiary amines reduce toxicity (Fischer et al., 2003). The PRAN modified polymers destabilised the membranes to a greater extent than ACAN modified polymers, confirming that an increase in hydrophobicity improves membrane interaction, in agreement with previous literature (Narita et al., 2001). Though the hydrophobicity of our propionic anhydride modified polymers improves membrane interaction, clearly it is not high enough to cause appreciable cell lysis. Haemocompatibility is important since free polymer is liberated after release of DNA; minimising interactions with the blood components ensures that the host immune system is not stimulated and reduces clearance rate, thus increasing transfection (Zeng et al., 2007).

3.4. Ethidium bromide exclusion assay

Ethidim bromide is a fluorescent dye that intercalates within DNA base pairs. When polymers interact with DNA to form polyplexes, they condense the DNA which prevents the base pairs intercalating with ethidium bromide, leading to a decrease in fluorescence (Geall and Blagbrough, 2000). This assay is quantitative (Zugates et al., 2007); the magnitude of fluorescence reduction correlates to the strength of polymer-DNA interactions (Rungsardthong et al., 2001). Both PEI and modified polymers showed maximal condensation at 1:1 w/w DNA: polymer, comparable with literature results (Gabrielson and Pack, 2006). Thus, acylation does not seem to drastically alter the DNA condensation ability of PEI. Comparison of polyplexes of PEI, ACAN modified polymers and PRAN modified polymers showed that at 1:1 w/w DNA: polymer ratio, while polyplexes of both ACAN and PRAN modified polymers showed slightly higher fluorescence intensity than PEI, polyplexes of PRAN modified polymers showed lesser fluorescence intensity than polyplexes of ACAN modified polymers (Fig. 3). Also, as the degree of acylation increased, the polyplex ratio had to be decreased to reduce the fluorescence intensities, i.e. more polymer was required to compact DNA.

The presence of protonable nitrogens on PEI helps it to interact and bind effectively with DNA (Tang and Szoka, 1997), while the reduced charge and decreased concentration of amines in the modified polymers weaken interactions with DNA. The highly modified polymers 1 ACAN, 2 ACAN, 1 PRAN and 2 PRAN have fewer primary amines (Table 1), but they formed non-compact polyplexes which

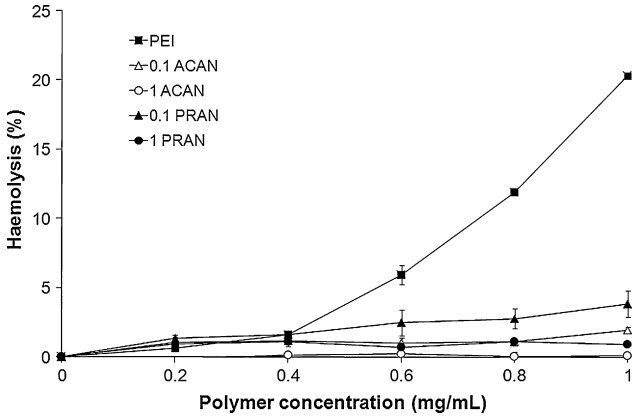

Fig. 2. Haemolysis percentage of PEI and modified polymers as a function of polymer concentration. Values are expressed as mean ± SD ($n = 3$); where error bars are not visible, they are within the symbols.

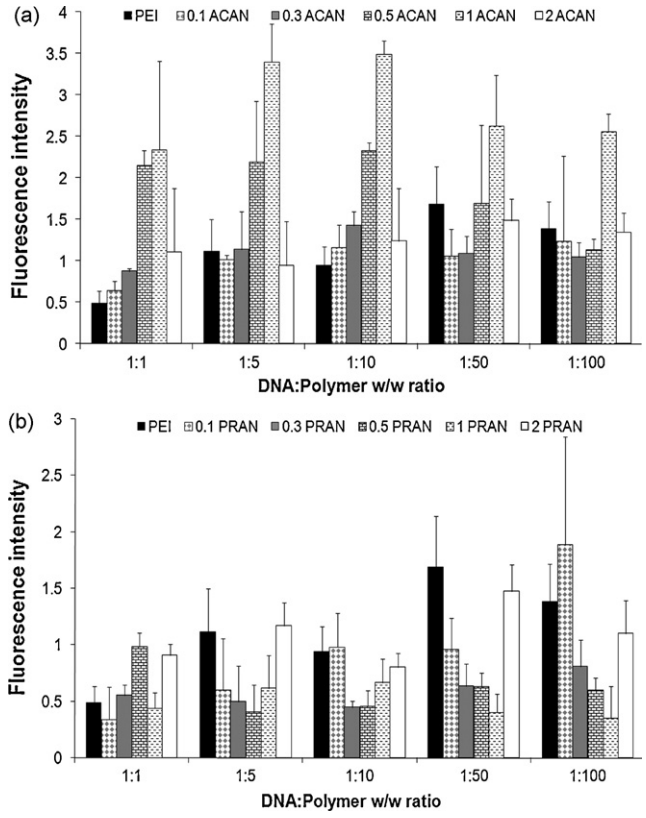

Fig. 3. Fluorescence intensity (mean ± SD, $n = 3$) as a function of varying DNA:polymer w/w ratios of (a) ACAN and (b) PRAN polymers. PEI and modified polymers show maximal condensation at 1:1 w/w DNA:polymer, but PRAN polymers show lower fluorescence intensities than ACAN polymers.

could be seen using transmission electron microscopy, implying that secondary amines are also involved in binding to plasmid DNA. However, these non-compact complexes did not transfect the cells in any of the three ratios tried, as the plasmid was not condensed effectively and was possibly digested by the DNAses (Pollard et al., 2001). While PEI interacts strongly with DNA, which helps condensation but hampers release of DNA in the cytoplasm, the reduced interactions of ACAN modified polymers might cause less efficient condensation and hence less compact complexes. The increased flexibility of the ethyl groups in the PRAN modified polymers (compared to the methyl groups in ACAN modified polymers) improves interaction with plasmid DNA and the intermediate strength of interactions might be optimal to cause both condensation of DNA and effective release of DNA in the cytoplasm. Also, the strength of polymer-DNA interactions influences the polyplex size and surface charge, which can alter toxicity and transfection.

Table 2
Size, polydispersity index and zeta-potential of the polyplexes.

Polyplex[a]	Size (nm)[b]	Polydispersity index	Zeta potential (mV)
PEI/DNA	107 ± 1	0.243	23.2 ± 0.4
0.1 ACAN/DNA	172 ± 1	0.404	23.7 ± 1.4
0.3 ACAN/DNA	114 ± 1	0.186	21.2 ± 0.8
0.5 ACAN/DNA	132 ± 3	0.105	17.0 ± 1.0
1 ACAN/DNA	548 ± 23	0.689	−36.6 ± 0.8
2 ACAN/DNA	640 ± 82	0.893	−37.8 ± 2.1
0.1 PRAN/DNA	117 ± 1	0.318	23.5 ± 0.7
0.3 PRAN/DNA	133 ± 4	0.174	21.1 ± 0.7
0.5 PRAN/DNA	168 ± 8	0.102	18.6 ± 1.9
1 PRAN/DNA	979 ± 148	0.336	4.69 ± 0.2
2 PRAN/DNA	456 ± 118	0.756	−30.3 ± 7.1

The values are expressed as mean ± SD ($n = 3$).
[a] (1:10 w/w DNA:polymer) prepared in PBS.
[b] The sizes indicated have been obtained from the most abundant peak of size distribution curve.

3.5. Size and zeta-potential of polyplexes

In addition to buffering capacity and compaction of the polyplexes, their size and zeta-potential affect passage through the cellular membrane. PEI formed the smallest polyplex, when compared to acylated PEI derivatives (Table 2). Though there is no clear trend between degree of acylation and size, it is clear that both ACAN and PRAN modified polymers formed relatively smaller particles at low degrees of acylation and larger particles at high degrees of acylation. The count rate and particle size increased for the polyplexes of highly modified polymers suggesting that the particles aggregate. They also had a high polydispersity index, which may indicate that compact polyplexes had not formed.

It has been reported that primary amines are required to form complexes with DNA (Tang and Szoka, 1997), so it might be expected that the highly modified polymers do not form polyplexes. However, transmission electron microscopy showed that particles were produced in these cases. The particles were very dense and staining was not required to see the images. PEI and polymers of low modification formed spherical particles while the highly modified polymers showed particle aggregation and irregularly shaped particles (Fig. 4). Microscopy also showed varied particle sizes, which were consistent with the size distribution data obtained from dynamic light scattering experiments. Smaller particles extravasate easily and distribute better within the cells and tissues (Wolfert et al., 1999). We expect that the smaller particles enter the cell more easily for transfection, while the larger particles are sterically hindered, leading to a decrease in transfection efficiency. Thus, controlling the size of polyplexes and preventing aggregation could enhance transfection.

The zeta potential of DNA was – 54.3 ± 3.63 mV. Acylation of PEI decreased the positive charge of the polymers and the zeta potential of the corresponding polyplexes. The decrease in zeta potential with increasing acylation suggests that DNA is less compacted and

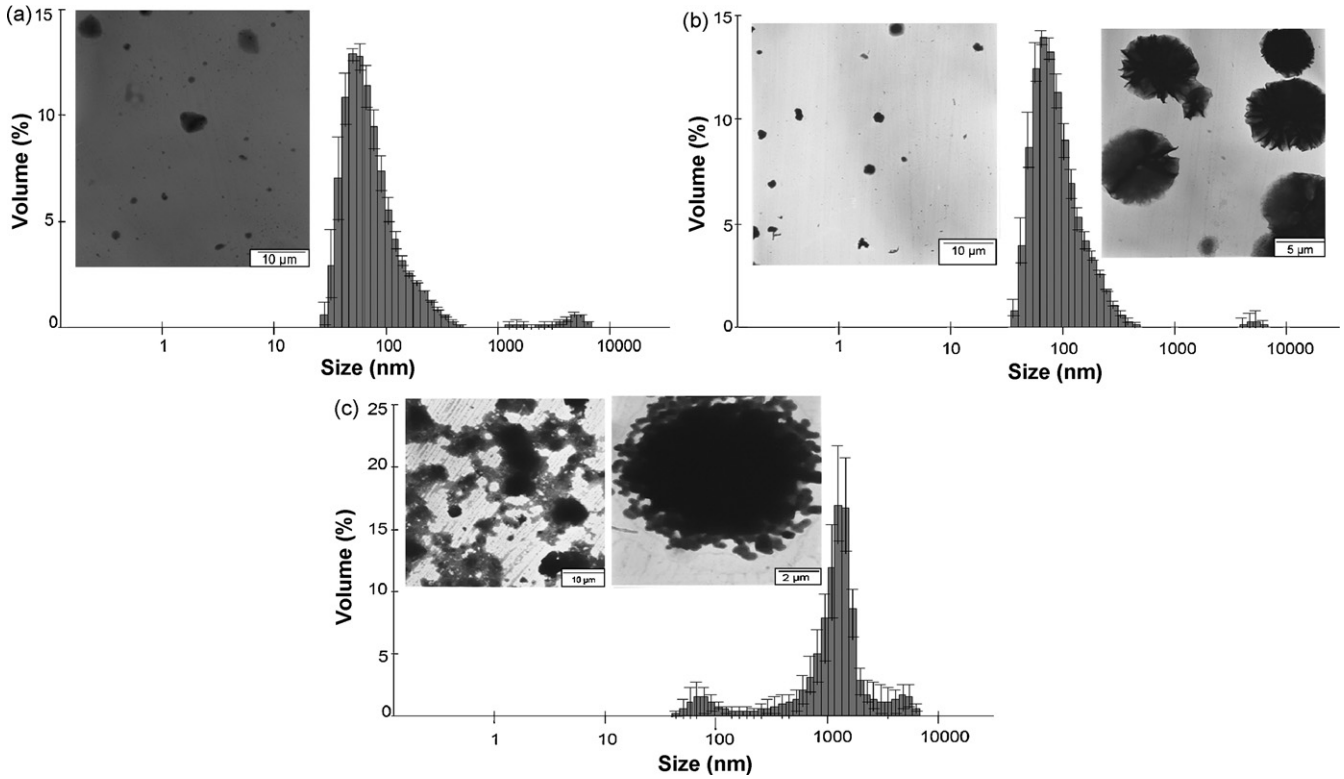

Fig. 4. Size distribution curves of polyplexes of (a) PEI, (b) 0.3 ACAN and (c) 1 ACAN at 1:10 w/w DNA:polymer ratio. Inserts show transmission electron microscopy images of the respective polyplexes.

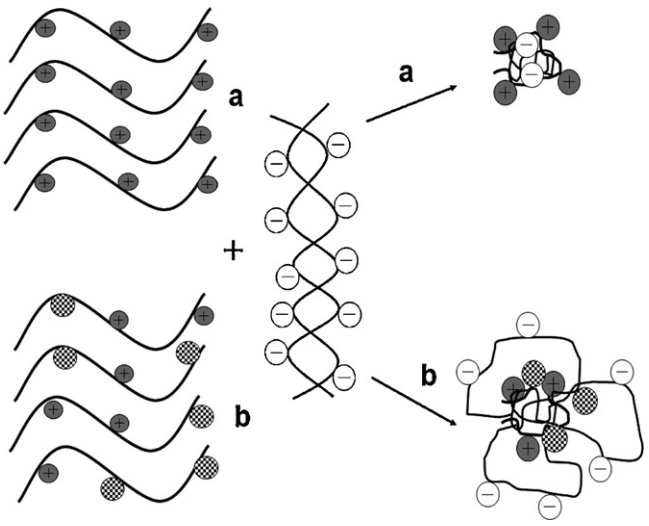

Fig. 5. Scheme of formation of polyplexes (a) PEI or PEI with low degree of modification forms compact polyplexes (b) PEI with high degree of modification forms less-compact polyplexes.

might expose more negative charge on the outside of the polyplex (Table 2). As long as the degree of acylation is low, there is good compaction of the DNA by the polymer and the positive potential causes electrostatic attraction with the cellular membrane, which enhances entry of polyplex into the cell. With increasing acylation, there is more negative charge on the outside of the polyplex, due to inefficient compaction, which hampers cell entry (Fig. 5). Also, when DNA is not compacted sufficiently, it might be prone to digestion by nucleases (Pollard et al., 2001). It is interesting to note that while polyplex of 1 ACAN shows a negative zeta potential, polyplex of 1 PRAN shows a positive zeta potential. Both the modified polymers had comparable concentrations of acylated primary amines. This confirms the results of our ethidium bromide exclusion assay, which showed that the strength of interactions of PRAN modified polymers with DNA was more optimal to cause condensation than that of ACAN modified polymers.

3.6. Cytotoxicity

The cytotoxicity of the polymers was investigated using MTT assay. The cells were incubated with polymer solutions for 6 or 24 h; the two time periods were chosen to mimic conditions required for *in vitro* and *in vivo* experiments. Complexing the cationic polymer with DNA reduces its toxicity, but the free polymer is liberated after polyplex dissociation in the cell. The polymer might exert its toxic effects until it is cleared or degraded by the cell, which might take 6 to 24 h depending on *in vitro* or *in vivo* conditions. PEI showed IC_{50} values of 17 μg/mL and 30 μg/mL after 24 and 6 h incubation with HEK293 cells. The modified polymers showed higher IC_{50} values and cytotoxicity decreased with increasing degree of acylation at both the incubation periods (Table 3). The IC_{50} of all the modified polymers, except 0.1 ACAN and 0.1 PRAN at 6 h, showed a significant difference when compared to the unmodified polymer (Table 3). Increasing the incubation period with the cells increased cytotoxicity; the increase was statistically significant for unmodified polymer ($p < 0.01$) and polymers of low modification 0.1 ACAN ($p < 0.01$), 0.1 PRAN ($p < 0.01$) and 0.3 PRAN ($p < 0.05$).

Cationic polymers such as PEI are toxic due to their strong interactions with plasma membrane (Choksakulnimitr et al., 1995) or interactions with negatively charged cell components (Morgan et al., 1989; Fischer et al., 2003; Fogera et al., 2006). Indeed, PEI showed the highest toxicity among a series of polycations tested in L929 mouse fibroblasts and the effect depended upon exposure time and

Table 3
IC_{50} values of PEI and derivatives at 6 and 24 h incubation in HEK293 cells.

Sample code	IC_{50} (6 h) (μg/mL)[a]	IC_{50} (24 h) (μg/mL)[a]
PEI	30.5 ± 3.54	17.0 ± 1.73
0.1 ACAN	32.8 ± 0.35	22.3 ± 1.15*
0.3 ACAN	40.2 ± 1.26*	36.7 ± 5.03**
0.1 PRAN	35.2 ± 1.04	25.3 ± 3.21*
0.3 PRAN	42.5 ± 0.87**	33.0 ± 4.58**
0.5 PRAN	47.8 ± 1.89**	46.7 ± 4.16***

[a] The values mean ± SD were calculated from cell viability percentages obtained from MTT assay done in triplicate and repeated three times. Statistical significance of modified polymer compared to unmodified polymer using two-sample *t*-test is given.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

concentration (Fischer et al., 2003). Our modified polymers have reduced surface charge, which could explain their reduced cytotoxicity. Studies on poly(L-lysine) and PEI showed that primary and secondary amines increased toxicity, while tertiary amines reduced toxicity (Fischer et al., 2003; Lv et al., 2006). Reducing the primary amine content of our polymers thus lowered their toxicity compared to PEI; cytotoxicity decreased with increasing degree of acylation because of the decreasing concentration of primary amines of the polymer. Increasing the incubation period with cells allowed the primary amines to exert their toxic effects; this explains the significant increase in cytotoxicity of PEI and the modified polymers with higher concentration of primary amines.

Complexing with DNA was expected to further improve the biocompatibility of our polymers, as DNA would neutralise some of the cationic charge on the polymer. However, at 1:1 DNA:polymer ratios, there was no difference in cell viability upon treatment with polyplexes compared to the modified polymer alone (Fig. 6a). Indeed, for the low modification polymers 0.1 ACAN and 0.1 PRAN, polyplexes surprisingly reduced cell viability when compared with the free polymers ($p < 0.001$). The reasons for this anomaly are at present unclear but may relate to localised charge density on these two polyplexes. In contrast, at 1:2 ratios, the polyplexes formed by modified polymers were, as predicted, significantly less toxic than their free polymers (Fig. 6b). Thus, in terms of minimising toxicity, a 1:2 w/w system seems to be the favoured ratio for the modified polymers to form polyplexes.

3.7. Transfection

The transfection efficiency of PEI and modified PEI was studied in HEK 293 cells. The polymers were complexed with EGFP to form polyplexes at three different ratios (1:1, 1:2 and 1:10 w/w DNA: polymer). The three ratios were chosen to test the effect of polymer concentration in the polyplex on transfection efficiency; at 1:1 compositions, the polymers formed compact polyplexes, 1:2 provided slight excess of polymer to give surplus cationic charge to the polyplex, while 1:10 provided large excess of polymer and cationic charge. During the experiments, the polyplexes were incubated with the cells for either 6 or 24 h to mimic *in vitro* and *in vivo* conditions. The highly modified polymers did not transfect the cells at any of the three ratios tried, irrespective of the incubation period. They formed larger polyplexes and/or had low or negative zeta potential, which might decrease the ability of the polyplexes to interact with and penetrate the cell membrane. Also, flexible molecules interact better with the cell membrane (Fischer et al., 2003). The polymers with a high degree of modification might be less flexible, which might hamper their interactions with the cell membrane, thus partially accounting for their lack of transfection. The high degree of acylation decreased the concentration of protonable nitrogens of the polymers and hence their buffering

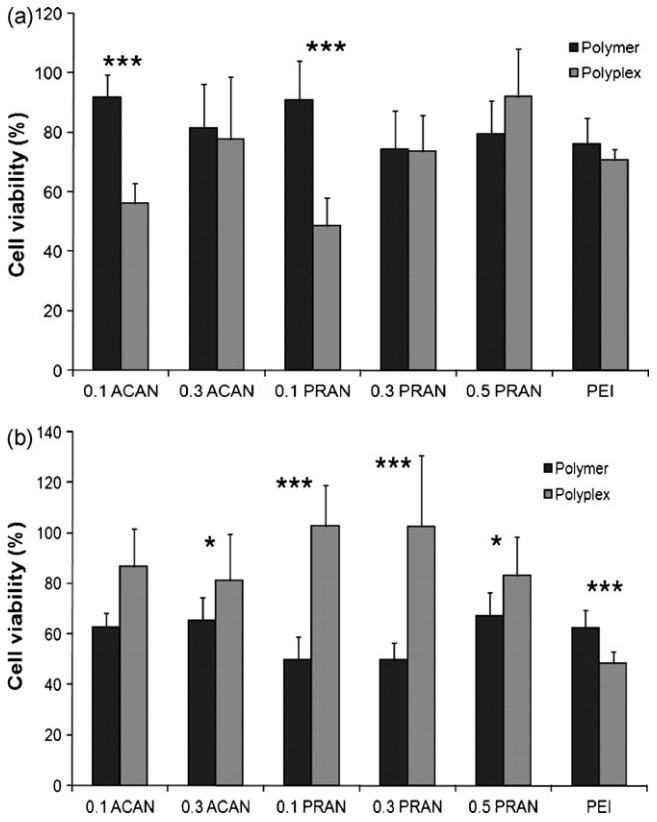

Fig. 6. Cell viability of polymers vs polyplexes at (a) 1:1 w/w DNA:polymer ratio and (b) 1:2 w/w DNA:polymer ratio. HEK293 cells were incubated with polymers or polyplexes for 24 h. Values are expressed as mean ± SD ($n = 8$). Statistical significances shown represent difference between cell viability of polymer and polyplex. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

capacities, which could have prevented endosomal escape. The modified polymers 0.5 ACAN and 0.5 PRAN had comparable buffering capacities, their size and zeta-potential was also similar; yet, 0.5 ACAN did not transfect the cells in any of the three ratios we tried, whilst 0.5 PRAN transfected the cells. Ethidium bromide exclusion assay showed that the fluorescence intensity of 0.5 ACAN was higher than 0.5 PRAN at the ratios studied. This implies that 0.5 ACAN interacted weakly with DNA and formed non-compact complexes, which accounts for its inability to transfect cells.

The polymers with a low degree of acylation showed transfection efficiencies varying with the polyplex ratio and incubation period. At 1:1 polyplex ratio, the modified polymers did not show any significant increase in transfection efficiency compared to unmodified PEI (Fig. 7a). The ethidium bromide fluorescence assay showed that a 1:1 w/w DNA:polymer complex was very compact. The effective condensation might prevent polyplex dissociation and the plasmid might not be available for the transcription machinery of the cell; when incubated with the cells for 6 h, PEI and the modified polymers showed transfection efficiencies less than 10%. Increase in the incubation period significantly increased transfection efficiencies of PEI, 0.1 ACAN and 0.1 PRAN, suggesting that improved cellular uptake and high buffering capacity might help to balance the limitations posed by polyplex dissociation, just by increasing the concentration of polyplexes in the cell cytoplasm. The transfection efficiencies decreased with increasing acylation of PEI. Both polyplex dissociation and endosomal escape were limiting factors for the modified polymers because of the reduced concentration of protonable nitrogens and improved DNA compaction (Erbacher et al., 1997). Most of the amines present on the polymer might be involved in DNA binding and compaction and there might not be enough protonable nitrogens on the polymeric chain to aid endosomal escape.

At 1:2 polyplex ratios, PEI and the modified polymers showed higher transfection efficiencies than at 1:1 ratios. At this composition, the polyplexes were not very compact due to the slight excess of polymer, which would have helped polyplex dissociation, thereby increasing transfection efficiencies. The polymers with a low degree of modification generally showed significantly increased transfection efficiency over unmodified PEI at both incubation periods, however as modification degree increased, transfection efficiency decreased (Fig. 7b). Also, the slight excess of cationic charge provided by the surplus polymer might improve interaction with cell membranes. Boeckle et al. (Boeckle et al., 2004) suggested that a small amount of free polymer enhances cellular uptake and subsequently improves transfection. However, further increase in modification degree, decreased transfection efficiency, as buffering capacity was not optimal to aid endosomal escape. An increase in the incubation period increased the transfection efficiencies of PEI and the modified polymers, because of improved cellular uptake. Both 0.3 ACAN and 0.3 PRAN showed significantly higher transfection efficiencies than PEI, because of a combination of factors including improved cellular uptake, optimal buffering capacity to facilitate endosomal escape and polyplex dissociation in the cytoplasm due to formation of less compact complexes.

At 1:10 polyplex ratios, the modified polymers did not show any significant increase in transfection efficiency compared to unmodified PEI and the transfection efficiencies were lower than those seen at 1:2 polyplex ratios (Fig. 7c). This could be because the excess polymer merely contributed to toxicity by increasing the surface charge and the concentration of primary amines in the vector. Only 0.1 PRAN showed significantly higher transfection than unmodified PEI after 6 h of incubation with the cells. Comparison of the two incubation periods showed that the low modified polymers 0.1 ACAN and 0.1 PRAN showed significantly higher transfection efficiency at 24 h incubation than 6 h, which could again be because of the improved cellular uptake.

Thus, of the three ratios tested, 1:2 w/w DNA:polymer appears to be the favoured ratio in the case of our modified PEI, as it provided a balance between plasmid condensation and dissociation. Correlating the transfection results with size and zeta potential data, it is clear that polymers which formed polyplexes smaller than 200 nm, with a zeta potential greater than 20 mV mediated efficient transfection. It has been suggested that red blood cells serve as a good membrane model to study vector interaction with cells and transfection (Brownlie et al., 2004). It could be argued that lack of haemolysis of our modified polymers suggests poor membrane interaction and low transfection efficiency. However, we found that the modified polymers were less haemolytic than PEI and those smaller than 200 nm with a zeta potential greater than 20 mV were more efficient transfection agents. Thus, in our case, red blood cells did not serve as a good model to predict the ability of the polymer to cause transfection. Also, it has been reported that transfection and toxicity varies between cell types (Florea et al., 2002). PEI does not release DNA efficiently into the cytoplasm (Godbey et al., 1999b). The reduction in surface charge of the modified polymers helped to form less compact polyplexes that could be easily unpacked within the cell, thus increasing transfection and reducing toxicity. The strength of polymer-DNA interactions is a crucial factor in gene delivery and the dissociation of plasmid from the polyplex limits gene transfer efficiency (Erbacher et al., 1996; Schaffer et al., 2000). Previous reports showed that buffering capacity did not correlate directly with gene delivery activity (Funhoff et al., 2004; Gabrielson and Pack, 2006). We found that buffering capacity in the range 3–9 μL/pH unit between pH 4.5 and 7.5 showed more efficient gene transfer than PEI (Fig. 8). Normally,

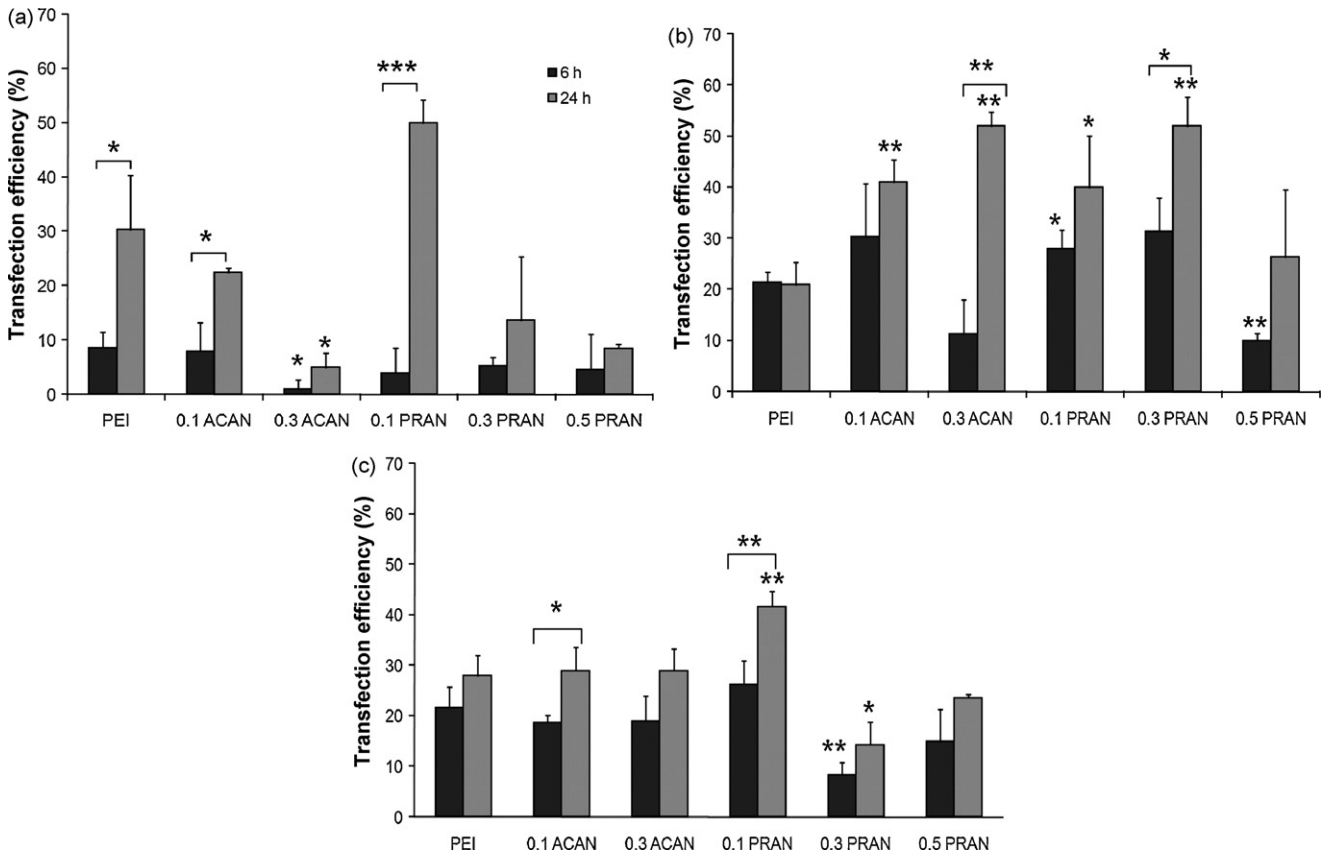

Fig. 7. Transfection efficiency of PEI and PEI derivatives of low modification; high modification derivatives did not give any transfection. Polyplexes were formed at (a) 1:1, (b) 1:2 and (c) 1:10 w/w DNA to polymer. Values are expressed as mean ± SD ($n = 3$). Statistical significances shown above cross bars represent differences between the incubation periods while those shown above individual bars represent differences between individual polymer with PEI at the same incubation period and polyplex ratio. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

it would be expected that a reduction in buffering capacity would prevent endosomal escape and thus lower transfection, but the low modified polymers showed higher transfection than PEI. Polymers with a lower buffering capacity were probably unable to escape from the endosomes and were cleared rapidly, while polymers with higher buffering capacity formed very stable complexes with the plasmid and did not release it efficiently in the cytoplasm, leading to decreased transfection efficiency. Transfection is a complex phenomenon and the reduced buffering capacity could be compensated for by the easy unpacking of the polyplex within the cell, as proposed earlier by Forrest et al. (Forrest et al., 2004; Gabrielson and Pack, 2006) or by the increase in the proportion of secondary amino groups, which may improve DNA compaction (BrissaultT et al., 2003). Even single carbon changes in pendant groups seem to affect polymer-DNA interactions by altering their physical properties and subsequent cellular uptake and transfection. Wang et al. showed that blocking primary amines and conjugating through secondary amines was not effective in increasing transfection efficiency (Wang et al., 2002). This is in agreement with our work where the highly modified polymers with a reduced percentage of primary amines did not show any transfection.

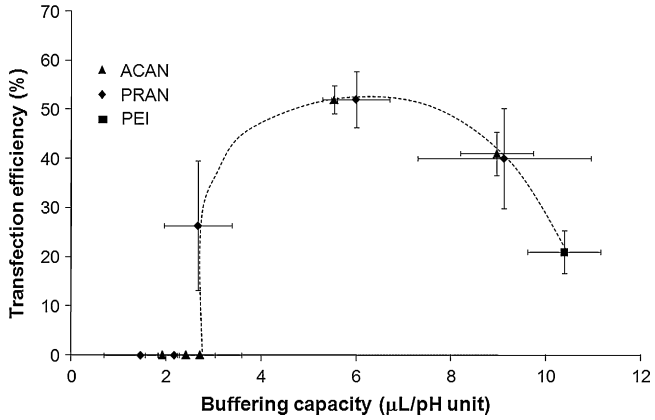

Fig. 8. Transfection efficiency of 1:2 compositions (after 24 h incubation) as a function of buffering capacity. The line is given as a general guide to show the trend. Polymers with buffering capacity in the range 3–9 μL/pH unit between pH 4.5 and 7.5 show more efficient gene transfer than PEI.

Our best vectors were 0.3 ACAN and 0.3 PRAN in a 1:2 w/w DNA:polymer composition. They showed significantly higher transfection efficiencies than unmodified PEI and were very effective when incubated with the cells for 24 h. The results of pH titrations and ethidium bromide exclusion assay show that the strength of interactions of PRAN modified polymers with DNA is optimal for DNA binding as well as release of DNA. Also, the haemolysis assay showed that PRAN modified polymers interacted better with the cell membrane, which could improve cellular uptake. Both the vectors showed comparable IC_{50} values, but when complexed with the plasmid, polyplexes of 0.3 PRAN showed significantly higher cell viability compared to 0.3 ACAN, making it a more attractive choice. Taking both efficient gene transfer and reduced toxicity into consideration, 0.3 PRAN is a more competent vector.

4. Conclusion

The amino groups on PEI backbone were acylated using acetic anhydride or propionic anhydride and the toxicity and transfection

efficiencies of the modified polymers were evaluated in HEK 293 cells. The acylated polymers showed reduced buffering capacities in solution and lower fluorescence intensities in the ethidium bromide exclusion assay, because of the reduced number of amines available for protonation and DNA binding. The modified polymers were more haemocompatible and less cytotoxic and showed increased transfection efficiencies due to effective plasmid release in cytoplasm. Polymers with buffering capacities greater than 50% and less than 80% relative to PEI, showed higher transfection efficiencies than PEI. The propionic anhydride modified polymers showed better membrane interaction and optimal binding strength with plasmid DNA and so were superior transfection agents compared to acetic anhydride modified polymers. The vector 0.3 PRAN in a 1:2 w/w DNA: polymer composition was the best vector as it showed increased transfection efficiency and reduced cytotoxicity.

Acknowledgements

LA thanks the University of Reading for supporting her PhD studentship. The authors thank Dr. F. Greco, School of Pharmacy, University of Reading for her advice and guidance on the MTT assay and Mr. Peter Heath, School of Chemistry, University of Reading for help with the NMR studies. We also thank Prof. N. Tirelli, University of Manchester for allowing use of his zetasizer.

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