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PEI-based vesicle-polymer hybrid gene delivery system with improved biocompatibility

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Abstract

Wider use of the transfection agent polymer polyethylenimine (PEI) in vivo has been hampered by its toxicity. In order to examine whether material combining properties of polymers and lipid type of carriers would have improved characteristics, four PEI derivatives were synthesised: The methylation of the branched PEI (25 kDa) created a permanently charged quaternary ammonium derivative. Acylation of these backbones using pendant palmitic acid chains created amphiphilic PEI variants which formed nanoparticles or vesicles. Finally hydrophilic groups were added to the polymer backbone by PEGylation. The materials were characterised and their in vitro and in vivo properties were tested. The modifications improved the materials biocompatibility markedly when compared to the starting material but also reduced transfection efficiency. The material bearing ammonium and palmitoyl groups was 10× less toxic while retaining about 30% of the transfection efficiency in vitro. After intravenous administration in a mouse model the materials also gave rise to GFP transgene expression in the liver. The synthetic strategy altered complex physicochemistry and improved biocompatibility while maintaining in vitro gene expression for most formulations. The strategy of combination of complementary properties of cationic lipids and polymers into a hybrid material may also be applicable to other materials.

Keywords: Polyethylenimine; Drug delivery carriers; Physical chemistry; DNA plasmids; Self-aggregation; Quaternisation

1. Introduction

Since their initial use as gene delivery system polyethylenimine (PEI) polymers have been extensively tested in vitro and in vivo and have been found to be one of the most efficacious non-viral agents (Boussif et al., 1995). Most of the PEI formulations studied to date have been prepared using branched PEI of varying molecular weight (0.6–800 kDa), but a linear PEI of 22 kDa has also been examined. Polyplexes from higher molecular weight branched PEIs (70–800 kDa) were found to be more efficient in vitro (Boussif et al., 1995; Fischer et al., 1999; Godbey et al., 1999; Ogris et al., 1999) but on intravenous administration the smaller and linear PEIs (Goula et al., 1998a; Kunath et al., 2003) seem in general to be more efficient than branched PEI of 25 kDa PEI (Bragonzi et al., 1999; Goula et al., 1998b) or 50–750 kDa PEI (Abdallah et al., 1996; Li et al., 2000). More recently cholesteryl PEI derivatives have also been shown to transfect cells (Furgeson et al., 2002; Han et al., 2001). Despite its efficacy PEI has some disadvantages, namely the bias of biodistribution/transfection towards the lung (Bragonzi et al., 1999; Goula et al.,

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1998a) and its significant toxicity in vitro and in vivo.

We have previously demonstrated that the manipulation of the physicochemistry of water-soluble polymers such as glycolchitosan (Dufes et al., 2000; Uchegbu et al., 1998) or poly-(L)-lysine and poly-(L)-ornithine (Brown et al., 2000) creates hybrid materials for drug delivery and gene delivery with excellent biocompatibility. The modified polyamino acid systems were—in contrast to the parent polymer—also capable of transfection in the absence of endosomolytic agents such as chloroquine (Brown et al., 2003). The aim of the present study was to explore whether such modular modification strategies could also be applied to improve the biological properties of the more efficacious PEI polymers.

2. Materials and methods

2.1. Synthesis and characterisation

A schematic overview of the synthetic route is given in Fig. 1. All materials for the synthesis (unless stated otherwise) were obtained from Sigma–Aldrich Co., UK. Synthesis and characterisation methods of PEI derivatives with the exception of the quaternisation procedure are described in detail elsewhere (Brownlie et al., submitted). When creating PEG–PEI co-polymer derivatives the starting point was the reaction of branched PEI (MW 25 kDa) with methoxy-polyethylene-glycol *p*-nitrophenyl carbonate. Amphiphilic PEI derivatives were created by reaction of palmitic acid *N*-hydroxy succinimide with the PEG copolymer or unmodified PEI, respectively.

2.1.1. Quaternisation

Quaternary ammonium PEI (QPEI) was synthesised by addition of methyl iodide (1.3 ml), sodium hydroxide (114.4 mg) and sodium iodide (128.7 mg) to a dispersion of PEI (315 mg, MW ∼25,000) in 1-methyl-2-pyrolidinone (50 ml, 12 h) and stirring under a stream of nitrogen for 3 h at 36 °C. The product was purified using precipitation in ethyl ether, washing (ethyl ether, ethanol), and exhaustive dialysis (QPEI dissolved in water, QPEI–PA in 1:1 methanol/water) against water. The dialysate (iodide salt) was further purified over an anion exchange column (1 cm × 6 cm, Amberlite-93) and then freeze dried.

2.1.2. Characterisation

All PEI derivatives were extensively characterised using FT-IR, NMR, elemental analysis, TNBS, GPC/LLS as described previously (for a summary of results cf. Table 1). ^{1}H NMR analysis (with integration), ^{13}C correlation and HMBC spectroscopy experiments were performed on solutions of PEI in D_2O (AMX 400 MHz spectrometer, Bruker Instruments, UK). A Perkin Elmer 2400 analyser was used for elemental analysis.

2.2. Preparation of complexes

Plasmid DNA (pCMVbeta, Invitrogen; pEGFP-C1, Clontech) was purified using a QIAGEN endotoxin free Giga Plasmid kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The purity and identity of the plasmid was confirmed by agarose gel electrophoresis (Ausubel et al., 1998).

The polymers PEI and QPEI were water-soluble; the palmitoylated derivatives PEI–PA, PEG–PEI–PA, QPEI–PA as well as the cholesterol containing formulations were dispersed in water using probe sonication (cf. Table 1).

To prepare the DNA complexes a volume (typically 100–200 $\mu g\,ml^{-1}$) of the polymer solution (in 5% dextrose) was slowly added to an equal volume of DNA solution, and then mixed thoroughly. The sizes and zeta-potential (ζ) of the resulting complexes were measured using dynamic light scattering (DLS, Zetasizer3000, Malvern Instruments).

2.3. In vitro assays

2.3.1. DNA binding and condensation

The binding and complexation of the polymeric formulations to DNA (0.1 $mg\,ml^{-1}$) reduces subsequent DNA intercalation of ethidium bromide (0.4 $\mu g\,ml^{-1}$). As only the intercalated proportion of ethidium bromide fluoresces strongly ($\lambda_{ex} = 526$ nm, $\lambda_{em} = 592$ nm) (Le Pecq, 1971) DNA condensation can be expressed as percentage of fluorescence intensity (relative units = RU) relative to un-complexed DNA ($RU = F_{complex}/F_{DNA}$) with maximum condensation being equivalent to 10% of the intensity of free DNA (maximum).

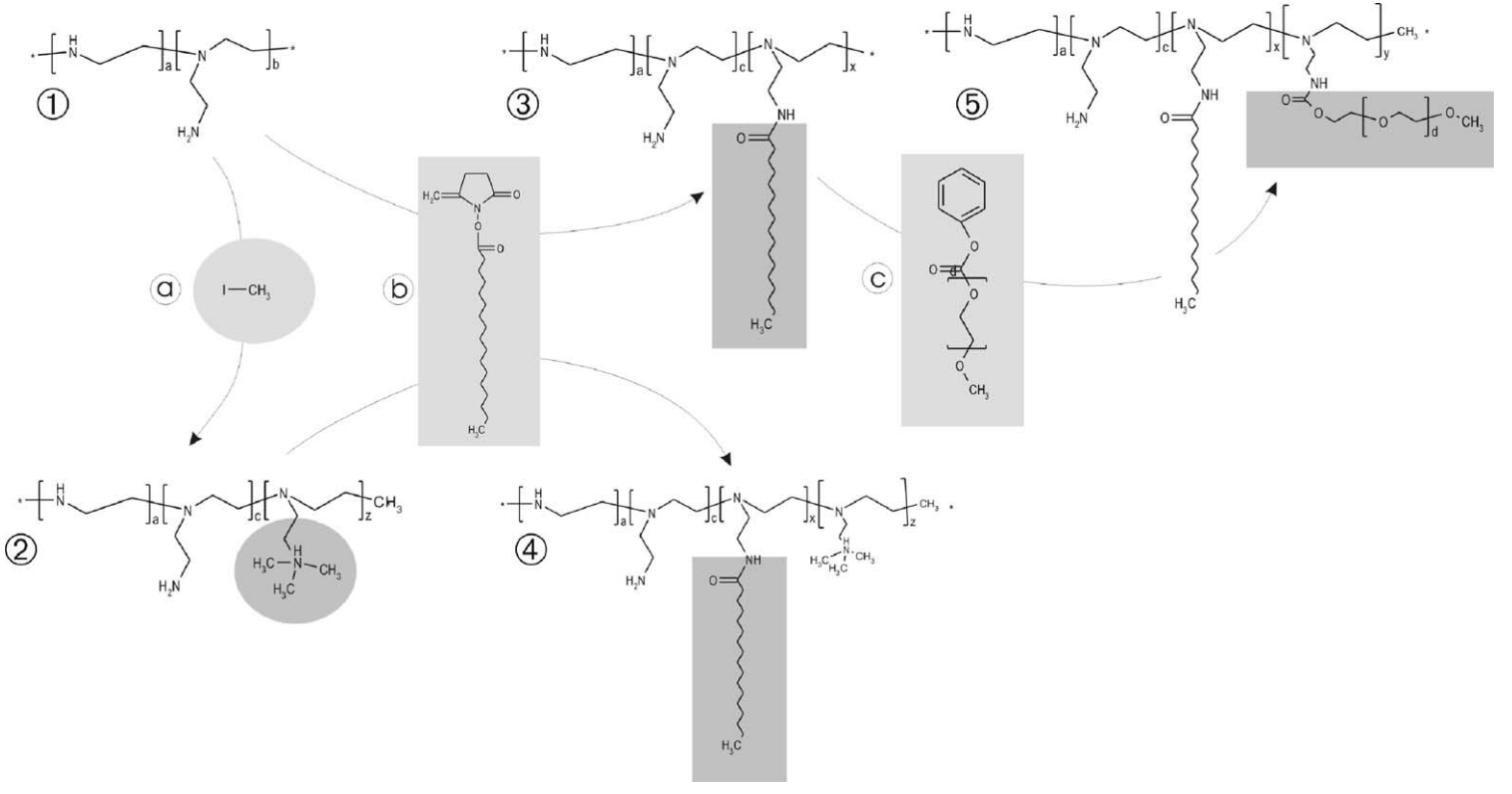

Fig. 1. Schematic diagram of PEI derivative synthesis. Reaction of the reactive amines of a branched 25 kDa PEI (①,$[\ldots]_b$) with methyl-iodide (ⓐ, "Q") created a quaternised PEI derivative (②, $[\ldots]_z$, "QPEI"). The covalent attachment of palmitic acid (ⓑ, "PA") using a reactive *n*-hydroxy group produced the respective amphiphilic comb-polymer derivatives of PEI (③, $[\ldots]_x$, "PEI–PA") and QPEI (④, $[\ldots]_x$, "QPEI–PA"). The amphiphilic PEGylated PEI co-polymer (⑤, $[\ldots]_y$, "PEG–PEI–PA") was synthesised by reaction with the side chain primary amine ($[\ldots]_b$) using methoxy-polyoxyethylene glycol (ⓒ, "PEG").

Table 1
Chemical and physicochemical characteristics of PEI and PEI derivative formulations (data other than for QPEI from Brownlie et al., submitted)

Derivative/ formulation	Modification	Nitrogen (mmol g^{-1} polymer)	Molecular weight	Solubility	Condensation ratio (polymer/DNA)	Zeta-potential (mV)	In vitro ratio (polymer/DNA)
PEI	233 units, 45 branches	22.9	23000 ± 850	Water-soluble	1:1 (w/w) 8:1 (N/P)	+35.2	0.5:1 (w/w)
PEI–PA	14% PA	10.9	46000 ± 2910	Particulate 169 nm	1:1 (w/w) 4:1 (N/P)	+30.5	1.25:1 (w/w)
PEG–PEI–PA	8% PA, 3.3% PEG	6.1	260000 ± 7700	Particulate 400 nm	2.5:1 (w/w) 5.5:1 (N/P)	+20.6	2:1 (w/w)
PEG–PEI–PA/Chol	2:1 Polymer/Chol (w/w)	–	n/a	Vesicular	5:1 (w/w) 11:1 (N/P)	+27.8	2:1 (w/w)
QPEI	26% Q	10.8	–	Water-soluble	1:1 (w/w) 3.6:1 (N/P)	+50.8	2:1 (w/w)
QPEI–PA	14% PA	<9.4	48000 ± 2350	Particulate 125 nm	2.5:1 (w/w) 6:1 (N/P)	+60.3	0.5:1 (w/w)
QPEI–PA/Chol	2:1 Polymer/Chol (w/w)	–	n/a	Vesicular 6:1 (N/P)	2.5:1 (w/w)	+75.8	2:1 (w/w)

Transfection was carried out according to a previously published method (Brown et al., 2000) for a range of polymer/DNA ratios.

2.3.2. Cytotoxicity

The lung carcinoma cells A549 (ATCC CCL-185) and the epidermoid carcinoma cells A431 (ATCC CRL-1555) were incubated with varying concentrations of the PEI derivative formulations for 4 h. Then media were replaced and relative cell numbers measured after 72 h using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue) assay and expressed relative to negative (PBS) and positive (Triton X-100, 1%, w/v) controls (Plumb et al., 1989).

Haemolysis assays were carried out essentially as published previously (Brown et al., 2000). Briefly, 3% (w/w) dispersions of washed human erythrocytes 3% (w/w) were mixed (1:1, v/v) with varying amounts of the derivatives in 96 well plates, incubated for 4 h (37 °C), and centrifuged (1000 RCF). Lytic activity was measured as absorbance (570 nm) of the supernatant (100 μl) and expressed as percent lysis relative to lysis induced by addition of PBS ("negative") and Triton X-100 (1% w/v, "positive") controls. To asses the potential to induce erythrocyte aggregation and erythrocyte aggregation (Ogris et al., 1999) suspensions were prepared as above. After the incubation period settled cells were mixed gently and aliquots transferred onto a slide for viewing.

2.4. In vivo experiments

DNA complexes (50 μg DNA per mouse) and controls were injected in a volume of 200 μl into the lateral tail vein of CD-1 mice (∼20 g, $n = 4$). Animals were killed 24 h later and the organs fixed in para-formaldehyde solution (4 °C, 24 h), embedded in paraffin and thin sections cut.

The slides were de-waxed, stepwise re-hydrated and then permeabilised in 0.05% saponin for 20 min. Immunohistochemistry was carried using polyclonal anti-GFP antibody (complete rabbit serum, Molecular Probes) and a peroxidase staining kit (Vectastain Elite ABC kit, Rabbit IgG, Vector Laboratories) according to the manufacturers' instructions. An additional blocking step using mouse serum (4.5 ml of BSA buffer + 0.5 ml mouse serum) was included

in the procedure. Slides were counterstained with haematoxylin, dehydrated and mounted. Complete cross-sections of the organ were captured as a series of three to five overlapping high-resolution micrographs (Zeiss Axiocam/Axiovision 3.0) at low magnification (2.5× Plan-Neofluar lens, Zeiss Axiophot microscope) with colour correction (cf. Fig. 5).

3. Results

3.1. Chemistry and physicochemistry of PEI graft-co-polymer derivative formulations

A series of novel graft-(co)polymer derivatives of PEI have been synthesised as schematically outlined in Fig. 1 (see also for abbreviations of compound names). Based on the branched polyethylenimine starting material three types of modular modifications were introduced, namely acylation using palmitic acid, PEGylation, and quaternisation, which we hypothesised, would modify the systems' physicochemistry and thus be potentially beneficial for gene delivery in vivo. The modular nature of the modifications has allowed us to create a number of combinations (Fig. 1), namely PEI, PEI–PA, PEG–PEI–PA, QPEI, and QPEI–PA.

The characterisation of PEI–PA, PEG–PEI–PA, and QPEI–PA polymers have been described in detail elsewhere (Brownlie et al., submitted). QPEI synthesis was confirmed by NMR which showed a downfield shift of the CH_2 peaks, indicating an increase in the electro-negativity of the backbone and terminal nitrogen of the polymer. The ^{13}C spectrum shows the presence of CH_3 peaks at both $\delta = 50$ and 52 ppm, which can be attributed to the quaternary ammonium groups, $N+(CH_3)_3$. The presence of tertiary amino methyl was confirmed by the ^{13}C NMR signal at $\delta = 40$ ppm.

Grafting of hydrophobic palmitoyl chains to the backbone modulated the self-aggregation capability of the water-soluble PEI backbone and created an amphiphilic polymer, which was able to form nanoparticles and vesicles when co-formulated with cholesterol (cf. Table 1). Methylation of PEI generated quaternised amines which carry a permanent cationic charge. The increased charge density is reflected in an decrease of the N:P ratio at which maximum DNA condensation[1] and thus increases the DNA binding capacity of the polymer (cf. Table 1). In addition the ζ-potential of the complexes was also increased, e.g. for PEI → QPEI from 35 to 50 mV and for PEI–PA → Q-PEI–PA from 30 to 60 mV (cf. Table 1).

The PEGylation of the amphiphilic PEI (PEI–PA) created a PEG–PEI–PA graft-copolymer which forms nanoparticles or vesicles (with Chol) with hydrophilic surface modifications. The sterically stabilised material has a reduced ζ-potential of +20.6 mV (PEG–PEI–PA) and 27.9 mV (PEG–PEI–PA/Chol), respectively.

By combination of these modular modifications of PEI and co-formulation with cholesterol (Chol) six formulations with distinct physicochemical properties were created with the aim of identifying potentially beneficial modifications. The chemical and physicochemical properties of the various formulations with and without cholesterol have been summarised in Table 1.

3.2. Biological properties of PEI-based carriers

In order to asses the biological effects of the modifications the formulations were screened using in vitro assays and subsequently tested in a mouse model.

3.2.1. Modified PEI-based synthetic delivery systems have a relatively low transfection activity in vitro

In vitro transfection was tested over a broad range of polymer/DNA ratios, i.e. from 80:1 (w/w) to 1:6.4 (w/w) in A431 and A549 cells. The optimum carrier/DNA ratio for the cationic lipid control (DOTAP) and the PEI polymer control were consistent with literature values, i.e. 5:1 and 1:2, respectively (cf. Fig. 2). The modified PEI formulations had a consistently lower efficiency than both, the parent polymer and DOTAP. In both cell lines the hybrid system PA-PEI formed the most efficacious complex with maximum expression levels between 30 and 40% of the activity of the parent polymer and about 30% of the DOTAP activity. The other PEI derivative complexes were significantly less active. The trend QPEI > QPEI–PA/Chol > QPEI–PA > PEG–PEI–PA was simi-

[1] Defined as 10% of the initial ethidium bromide fluorescence.

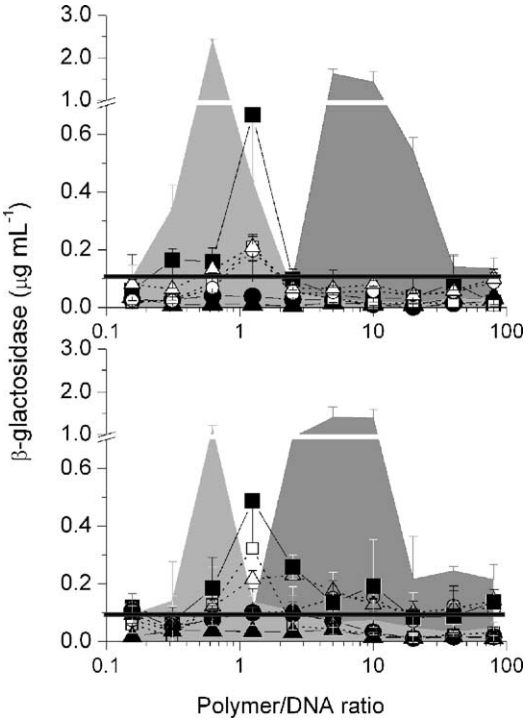

Fig. 2. Transfection efficiency of PEI-based complexes in vitro comparing β-galactosidase transgene expression after transfection of A431 and A549 cell lines with pCMV β-gal, alone and complexed in PEI hybrid systems at various ratios. For both cell lines (A549, top panel and A431, bottom panel) the controls, represented by small symbols and filled in traces (PEI, light filled trace; DOTAP, dark filled trace), naked DNA background levels are indicated by the black, horizontal line. PEI derivatives carry large filled symbols, the symbols for quaternised derivatives are outlined only (PEI–PA (■), PEG–PEI–PA (●), PEG–PEI–PA/Chol (▲), QPEI (□), QPEI–PA (○), QPEI–PA/Chol (△)). A break in the axis between 0.7 and 1.0 μg ml^{-1} was introduced to improve readability at lower expression levels.

lar in both, A431 and A549 cells, but overall expression was lower in the fomer. Interestingly the polymer/DNA ratios that gave rise to the most active complexes were similar to the minimum polymer/DNA ratios that still gave full condensation and yielded colloidally stable complexes (cf. Fig. 2).

3.2.2. PEI derivatives have an improved toxicity profile

The cytotoxicity of synthetic vectors can be a problem and frequently limits the total amount of DNA that can be administrated safely. While the branched 25 kDa PEI itself has an IC_{50} of 5.2 μg ml^{-1} (A549) and 1.9 μg ml^{-1} (A431), respectively, most derivatives are considerably less toxic than the parent polymer (cf. Table 2). Although the general ranking of formulations was similar, considerable differences in toxicity exist between both cell lines with IC_{50} values ranging form PEI–PA in A549 cells (4.9 μg ml^{-1}), which in this cell line is as toxic as the starting material PEI, into the thousands of micrograms for the complexes of the PEGylated compounds with DNA (cf. Table 2).

In A431 cells the quaternisation of PEI to QPEI moderates the toxicity more than four-fold; additional palmitoylation (QPEI–PA) reduces the toxic effect by a factor of more than eight-fold whether formulated as nanoparticles or vesicle with the help of cholesterol. While in the A431 cells the palmitoylation alone (PEI–PA) halves the toxicity compared to PEI it has no effect in A549 cells. A549 cells are less sensitive to the parent polymer than A431 cells ($IC_{50} = 5.2$ μg ml^{-1} versus $IC_{50} = 1.9$ μg ml^{-1}) but also benefit relatively less from most modifications (cf. Table 2).

A very strong effect is seen after PEGylation which yielded similar reductions of toxicity in both cell lines. The addition of PEG to the PEI–PA graft-polymer reduced the toxicity from 4.9 μg ml^{-1} (PEI–PA) to 58 μg ml^{-1} (PEG–PEI–PA) and from 3.98 μg ml^{-1} (PEI–PA) to 19 μg ml^{-1} (PEG–PEI–PA) in A549 and A431 cells, respectively.

In addition to assessing the toxicity of the polymer materials alone complexes with DNA were also evaluated and found to be consistently less toxic than the polymer alone. The reduction of toxicity observed ranged from 1.4-fold to more than 10-fold. In contrast to the PEI derived materials, where the bigger improvement was observed in the more sensitive A431 cells, the effects as far as the complexes were concerned were stronger for the less sensitive A549 cell line (cf. Table 2).

3.2.3. Modified polymers have improved haemocompatibility

The interaction of the PEI-based materials with blood components and the colloidal stability of the respective DNA complexes was tested by examining their ability to induce lysis and aggregation of erythrocytes (cf. Figs. 3 and 4).

Haemolysis is a potential side effect linked to the intravenous administration of synthetic gene delivery

Table 2
Absolute and relative cytotoxicity (IC_{50}) of PEI and PEI derivative formulations with and without complexation to DNA

Cell line	IC_{50} ($\mu g\,ml^{-1}$)				IC_{50} (relative)			
	Polymer		Complex		Complex vs. PEI		Complex vs. carrier	
	A549	A431	A549	A431	A549	A431	A549	A431
PEI	5.2	1.9	13.3	3.6	1.00	1.00	2.56	1.89
PEI–PA	4.9	3.98	27.8	5.6	0.94	2.09	5.67	1.41
PEG–PEI–PA	58	19.9	>1 mg	68.1	11.15	10.47	>10	3.42
PEG–PEI–PA/Chol	61	20.4	>1 mg	80.4	11.73	10.74	>10	3.94
QPEI	7.7	8.6	32.3	24.2	1.48	4.53	4.19	2.81
QPEI–PA	12.6	16.9	26.6	37.8	2.42	8.89	2.11	2.24
QPEI–PA/Chol	11	15.9	28.5	34.1	2.12	8.37	2.59	2.14

systems that contain membrane active components, in particular amphiphiles. It is also indicative of the tendency of these carriers to interact with (and thus disturb) other cellular membranes. This tendency of the various PEI derived DNA complexes was examined using the lysis of erythrocytes as a model system (Fig. 3).

The cationic lipid DOTAP with its detergent-like structure causes substantial erythrocyte haemolysis (87% at $1\,mg\,ml^{-1}$). The parent polymer PEI is water-soluble but nevertheless shows a dose-dependent tendency to cause haemolysis (27% at $1\,mg\,ml^{-1}$). By comparison all PEI hybrid systems have an improved safety profile: both water-soluble and particle/vesicle forming derivatives cause less than 10% haemolysis at these concentrations. Only PA-PEI demonstrates a dose-dependent haemolysis above the background levels with a maximum of about 17% at $1\,mg\,ml^{-1}$, considerably lower than that of the parent polymer.

The interaction of DNA complexes with particulate/cellular elements in the blood stream was evaluated using the aggregation of erythrocytes as a model (Ogris et al., 1999). The assay allows a semi-quantitative evaluation and ranking of the tendency of various formulations to induce cellular aggregation

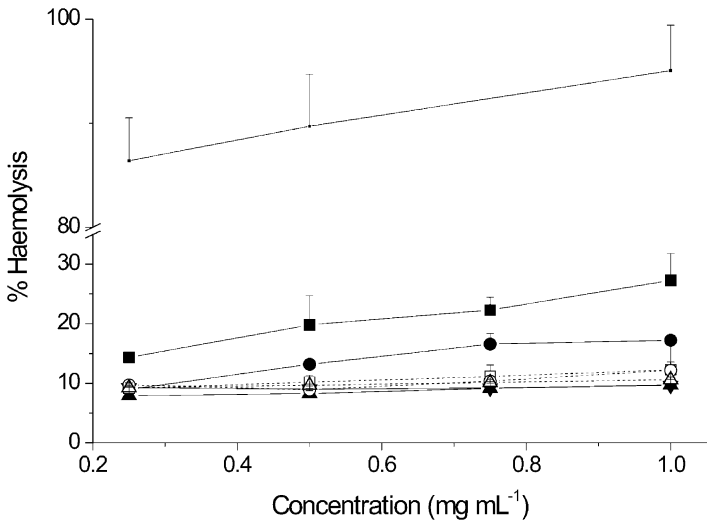

Fig. 3. Haemolytic activity of PEI derived polymer systems. Illustrated is the interaction and lysis of erythrocytes induced by various modified PEI carrier materials in comparison to PEI and DOPTAP controls (values for DOTAP vesicles are represented by a line only, PEI–PA (■), PEG–PEI–PA (●), PEG–PEI–PA/Chol (▲), QPEI (□), QPEI–PA (○), QPEI–PA/Chol (△)).

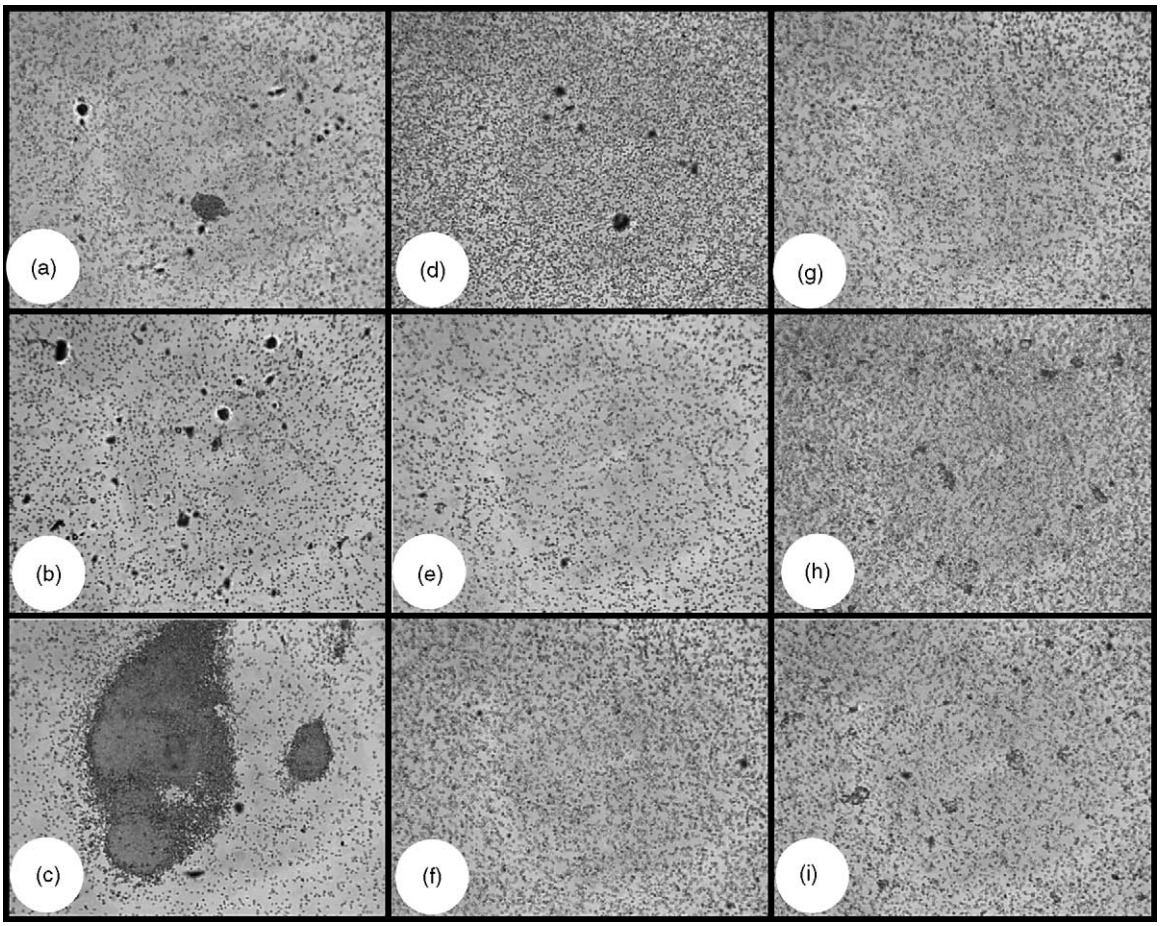

Fig. 4. Erythrocyte aggregation assay. Aggregation of erythrocytes caused by co-incubation of various DNA complexes of PEI hybrid systems and controls: (a) DOTAP, (b) PEI, (c) PEI (d) PEI–PA, (e) PEG–PEI–PA, (f) PEG–PEI–PA/Chol, (g) QPEI, (h) QPEI–PA, (i) QPEI–PA/Chol. (c) One of a large aggregate of potentially fused erythrocytes observed in the DOTAP and PEI controls.

(cf. Fig. 4). Both controls, DOTAP and PEI, induced a considerable amount of aggregation with the majority of aggregates being relatively small, i.e. between 5 and 10 erythrocytes in diameter (cf. Fig. 4a and b). There was some reversibility that allowed aggregates to re-disperse on shaking. In both cases there were also larger aggregates containing hundreds of cells (cf. Fig. 4c). The cell walls between adjacent cells were only partially visible suggesting the possibility of membrane fusion events. In the case of the PEI derivatives erythrocyte aggregation was always less than that observed with both, DOTAP and PEI, controls and no large aggregates were observed. The general trend observed for erythrocyte aggregation was DOTAP > PEI> QPEI–PA = QPEI–PA/Chol > PEI–PA > QPEI > PEG–PEI–PA = PEG–PEI–PA/Chol (cf. Fig. 4).

3.2.4. GFP expression in the mouse liver after systemic administration

In vivo transfection efficacy was assessed 24 h after intravenous injection of ‘naked’ DNA (plasmid in 5% dextrose) and various DNA-polymer complexes (50 μg DNA in 200 μl^{-1}) in mice. Liver sections were stained for GFP expression by immunohistochemistry (cf. Fig. 5) to avoid potential ambiguity due to the relatively high autofluorescence background in the liver. Histochemical staining of liver sections

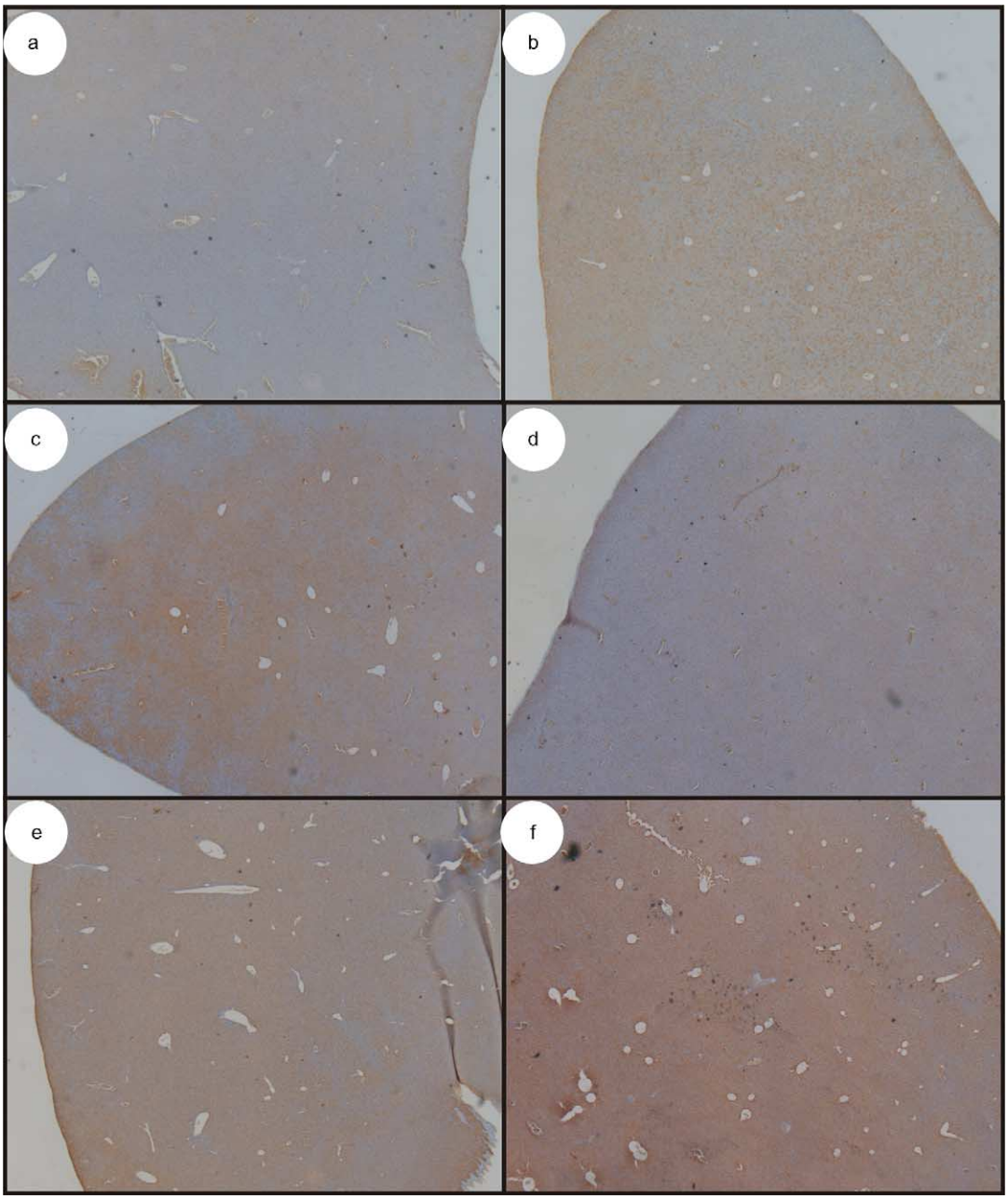

Fig. 5. GFP expression in the murine liver. Immunohistochemistry of GFP reporter gene expression in liver sections 24 h after administration of various formulations in mice: DNA only (a), PEI (b), PEI–PA (c), PEG–PEI–PA (d), QPEI (e), QPEI–PA (f).

was in general heterogeneous between animals but expression above background levels was evident for all formulations. The rank order PEI < PEG–PEI–PA < PEI–PA < QPEI < QPEI–PA emerged. For all formulations enhanced staining was sometimes observed at the centre of the liver lobules or, alternatively, in its periphery suggesting a potential influence of blood flow in the portal triads (not shown).

4. Discussion

One of the paradigms behind the development of synthetic vectors is their perceived safety in comparison to viral vectors. In particular for the use of genetic therapies for non-life threatening diseases it is crucial that the vectors have an excellent safety profile. Although 'safety' of liposomal vectors has been repeatedly attested in clinical studies (2002), animal studies suggest that a balance needs to be struck between efficacy and safety (Diebold et al., 1999). Life threatening side effects have been reported in mice when higher amounts of polymer-DNA complexes have been used (e.g. for PEI; Coll et al., 1999; Goula et al., 1998a; Ogris et al., 1999). Toxicity has in general been observed in vivo after PEI doses greater than 40–100 mg per mouse at N/P ratios (polymer amines to DNA phosphate) of 6:1 and greater (Goula et al., 1998a; Li et al., 2000; Ogris et al., 1999).

We have previously shown that the conjugation of palmitic acid with polylysine reduces the toxicity of this polymer while maintaining the transfection efficacy in vitro (Brown et al., 2000). We hypothesised that a similar strategy may also be able to improve the biocompatibility of PEI. Low molecular weight (MW = 1.8–2.0 kDa) PEI has previously been linked with lipophilic groups (Han et al., 2001; Kim et al., 2001; Oku et al., 1987; Thomas and Klibanov, 2002) but prior to our own work (Brownlie et al., 2000) PEI amphiphiles have not been reported to form colloidal, non-micellar (cloudy) dispersions or to order into bilayer vesicles (cf. Table 1). Furthermore, we speculated that the modifications could potentially also modulate in vivo expression patterns of PEI.

In vivo toxicity can result from the vector components, i.e. the toxicity of the polymer or of the DNA alone, but can also be related to the combination of both in a DNA complex. Results from the in vitro toxicity (cf. Table 2) and haemocompatibility studies (cf. Figs. 3 and 4) in this report indicate that the effects of the polymers alone and of the respective complexes are by no means interchangeable. The cytotoxicity of the polymer will in general be reduced after complexation to DNA but in vitro aggregation experiments suggest the tendency for aggregation in plasma or serum is frequently enhanced.

While the acute toxicity of linear 22 kDa PEI has been linked to its tendency to cause aggregation but also endothelial activation (Chollet et al., 2002) and thus is not necessarily due to mechanical blockage by large aggregates. Compromised colloidal stability in vivo may, however, still play a role for some formulations (own observations and Ogris et al., 1999) but toxicity also depends strongly on mouse strain and age of the animals (Chollet et al., 2002).

We have been able to demonstrate that the modular modifications of PEI altered the physicochemistry of the complexes but also modified the interaction of these hybrid systems with the elements of the vascular compartment. Specifically, the modified PEI carriers have a reduced tendency to aggregate or induce aggregation in serum, plasma (data not shown) and erythrocytes (Fig. 4). Both cationic lipid vesicles and the parent polymer PEI have a higher tendency to disrupt membranes than any of the modified materials as shown by the haemolysis assay (Fig. 3). Furthermore, their cytotoxic safety profile is significantly improved, e.g. in the case of PEG–PEI–PA by more than an order of magnitude, when compared to the parent polymer (Table 2). The loss of some binding activity could potentially be alleviated by replacement with specific interaction such as transferrin ligands (Brown et al., 2003; Li et al., 2003).

While the modifications clearly improved the safety profile they did not increase the transfection efficiency in vitro (Fig. 2). In a recent report similar observations were made for permethylated and hexadecylated branched PEI synthesised using a different synthetic strategy. In this study only the lipophilic modification of low molecular PEI (2 kDa) resulted in improved transfection activity in vitro (Thomas and Klibanov, 2002).

In the current study carriers with very similar composition but different supramolecular structure were examined. The initial supramolecular structure of the carrier, i.e. complexes formed from nanoparticles or vesicles, did not seem to have a clear biological effect. It is currently not clear whether these structural differences between the different particulates are lost during the complex formation or whether there is no differentiation in their cellular processing.

Taken together our results suggest that in order to develop efficient gene delivery systems a precarious balance needs to be struck between complex stability and DNA release. For polyplexes stability increases with the molecular weight of the polymer (multiple

charge interactions), in the case of cationic lipids complex formation depends on the aggregation of the amphiphilic monomers which bind DNA in mono–oligo valent fashion. Amphiphilic PEI derivatives combine multivalent interaction and self-assembly in one stabilised hybrid systems. Our results suggest that it may be advantageous to re-adjusted the balance between complex association and dissociation in favour of more stable complexes when delivery in vivo is being considered.

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