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Journal of Drug Targeting, May 2004 Vol. 12 (4), pp. 223–236

Taylor & Francis healthsciences

Genuine DNA/polyethylenimine (PEI) Complexes Improve Transfection Properties and Cell Survival

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Polyethylenimine (PEI) has been described as one of the most efficient cationic polymers for *in vitro* gene delivery. Systemic delivery of PEI/DNA polyplexes leads to a lung-expression tropism. Selective *in vivo* gene transfer would require targeting and stealth particles. Here, we describe two strategies for chemically coupling polyethylene glycol (PEG) to PEI, to form protected ligand-bearing particles. Pre-grafted PEG–PEI polymers lost their DNA condensing property, hence their poor performances. Coupling PEG to pre-formed PEI/DNA particles led to the expected physical properties. However, low transfection efficacies raised the question of the fate of excess free polymer in solution. We have developed a straightforward a purification assay, which uses centrifugation-based ultrafiltration. Crude polyplexes were purified, with up to 60% of the initial PEI dose being removed. The resulting purified and unshielded PEI/DNA polyplexes are more efficient for transfection and less toxic to cells in culture than the crude ones. Moreover, the *in vivo* toxicity of the polyplexes was greatly reduced, without affecting their efficacy.

Keywords: Transfection; Non-viral vector; Polyethylenimine; PEG; Gene delivery

INTRODUCTION

The cationic polymer polyethylenimine (PEI) has been described as one of the most potent non-viral gene delivery vectors in an increasing number of *in vitro*, as well as *in vivo* studies (Abdallah *et al.*, 1995; Boussif *et al.*, 1995; Goula *et al.*, 1998a; Coll *et al.*, 1999; Aoki *et al.*, 2001). PEI is used for systemic delivery (Goula *et al.*, 1998a; Bragonzi *et al.*, 2000; Zou *et al.*, 2000), and found that, linear PEI (L-PEI) was much more potent than branched PEI (B-PEI). However, ca. 95% of transgene expression was found in the lungs (Goula *et al.*, 1998a; Bragonzi *et al.*, 2000). The mechanism by which this "non-specific targeting" occurs is still under investigation (Goula *et al.*, 2000; Chollet *et al.*, 2001). As intravenous injection should be a route of choice for delivering genes in many therapeutic applications, several attempts, including ours, have been made to target *in vivo* ligand-derivatized PEI/DNA polyplexes to several organs or cell types (Erbacher *et al.*, 1999b; Li *et al.*, 2000). Although we were able to show that ligand-modified PEI/DNA polyplexes transfect the corresponding receptor-expressing cells *in vitro*, with efficacies much higher than those of native polymers (Kircheis *et al.*, 1997; Zanta *et al.*, 1997; Erbacher *et al.*, 1999b), none of these formulations showed positive results following intravenous injection. It is generally accepted that polyplexes for i.v. delivery should possess two well-defined physicochemical properties: (i) a size below 100 nm, in order to facilitate extravasation and diffusion within organs (Goula *et al.*, 1998b); (ii) a neutral/negative surface charge, to prevent RES clearance (Plank *et al.*, 1996; Ogris *et al.*, 1999). This implies to replace electrostatic interactions with the cell surface interactions, and to coat the complexes with a protective hydrophilic shell. Polyethylene glycol (PEG) is the most commonly used polymer for this purpose (Ogris *et al.*, 1999; Finsinger *et al.*, 2000).

We previously showed that polyethylene glycol grafting to branched PEI was interfering with proper DNA condensation into compact particles (Erbacher *et al.*, 1999a). To circumvent this problem, we describe here a *post-grafting strategy*, in which PEG or ligand–PEG residues are conjugated to preformed B- or L-PEI/DNA spherical polyplexes. Their physical characteristics (i.e. morphology, size and surface charge) were as anticipated, yet low transfection efficacies raised the question of excess free ligand–PEG–PEI polymer molecules that would compete with the polyplexes for receptor binding.

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ISSN 1061-186X print/ISSN 1029-2330 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10611860410001723487

We, therefore, separated the polyplexes from excess polymer and found that, at nitrogen per phosphate (N/P) ratios used for transfection, up to 50% of the cationic polymer was indeed free in the solution. Characterization of purified PEI/DNA polyplexes, as well as comparative *in vitro* toxicity and gene transfer data are described. Finally, a preliminary *in vivo* experiment using unshielded and purified polyplexes showed lower toxicity, without expense on vector efficacy.

RESULTS

Structure of Crude PEGylated PEI/DNA Complexes

B-PEI was pre-grafted with increasing PEG_{3400} amounts, to an extent corresponding to an equivalent of 0.5 to 4% of the PEI's total nitrogen atoms (2.5–20 PEG molecules per PEI). At any grafting extent, PEG–PEI conjugates lost their ability to properly condense DNA. Instead of condensing DNA, PEG–PEI polymers formed tangles of strings upon complexation with DNA (Fig. 1A). These particles, were electrostatically neutral and unable to transfect cells. With the aim of forming compact complexes, L-PEI was mixed with plasmid DNA prior to chemical coupling to PEG, by the formation of a carbamate linker (Bettinger *et al.*, 1998). As expected, transmission electron microscopy showed toroids and rods, but also loops extending out of condensed cores (Fig. 1B); the proportion of the later being related to the PEG grafting extent (data not shown). The same type of post-grafted particles was also prepared with B-PEI and showed relatively calibrated spherical structures, but with less loops (data not shown). Laser light scattering measurements, i.e. size and zeta potential, were in agreement with these observations, both showing small and neutral particles. However, optimized *in vitro* transfection using those post-grafted particles gave up to a hundred-fold higher reporter gene expression than the pre-grafted ones (10^4–10^7 RLU/mg proteins with a pCMV-luciferase encoding plasmid, HeLa cells, TB, unpublished results).

Aggregation Properties of Crude Post-grafted Polyplexes

Small L-PEI/DNA particles were prepared in 5% glucose solution (Goula *et al.*, 1998b). Albeit stable under this condition, the polyplexes aggregated if serum-albumin was added (Fig. 2A). Maximal aggregation was obtained between 1 and 2 mg/ml albumin. At higher concentrations, zeta potential became strongly negative (− 20 mV; data not shown) indicating that albumin had completely coated the particles and excess of protein prevented aggregation.

We then challenged particles towards aggregation induced by 1 mg/ml albumin and physiological saline concentration (Fig. 2B–D). Native L-PEI was found to be very sensitive to both conditions, with micron-range aggregates formed within minutes. PEG_{600} post-grafted polyplexes were still sensitive, although kinetics of aggregation were shifted to hours. Finally, PEG_{3400} post-grafted complexes became completely protected towards both salt- and protein-induced flocculation.

Complement System Activation

Post-grafted L-PEI/DNA particles were further challenged by an *in vitro* assay of complement system activation, described earlier (Plank *et al.*, 1996). In Fig. 3, %CH50max, which is inversely proportional to complement system activation was plotted against the PEI nitrogen concentration. Native L-PEI strongly activated the complement system. Increasing the molecular weight and/or the extent of PEG grafting reduced complement activation down to undetectable levels with 7% PEG_{3400}, independently of the chemical nature of the ligand grafted at the PEG distal end (RGD peptide vs. galactose).

Formulation of DNA with the Branched 25 kDa PEI into Small Particles

Plasmid DNA mixed with 25 kDa B-PEI formed large aggregates under physiological conditions (150 mM NaCl) at N/P ratio ≤ 5. Smaller particles (100–200 nm) were obtained at higher N/P ratios (10–20), but the size remained above 100 nm. Changing the complex formation medium to 20 mM HEPES buffer pH 7.4, significantly decreased both the size and polydispersity of the particles (Fig. 4).

Sedimentation Properties of PEI/DNA Complexes

When plasmid DNA was formulated with 22 kDa L-PEI in 5% glucose, small (30–100 nm) spheroidal particles were produced (Goula *et al.*, 1998b). We performed sedimentation experiments with ^{32}P-labeled DNA in conditions allowing the preparation of small particles either with the B- (in 20 mM HEPES pH7.4) or the L-PEI (in 5% glucose). PEI/DNA complexes made at several N/P ratio were centrifuged at various speed (20 to 10,000*g*) for 10 min. Irrespective of the N/P ratio, the amount of sedimented DNA was found less than 20 and 25% for polyplexes prepared with L- and B-PEI, respectively (Fig. 5A and C).

Purification of the PEI/DNA particles by ultrafiltration, required a high DNA concentration (200 μg/ml) and a low centrifugation speed (500 *g*). Three centrifugation cycles (10 min at 500 *g*) were performed on both L- and B- PEI complexes (Fig. 5B and D). The proportion of DNA found in the pellet was ≤ 20 and 30% at N/P ≥ 5, respectively.

Isolation of Genuine PEI/DNA Particles by Ultrafiltration:

Removal of excess free polymer in polyplex formulations, was performed by ultrafiltration through Centricon-100 filters. Control experiments showed that PEI/^{32}P-DNA complexes mostly ($>70\%$ at N/P ≥ 8) remained in the supernatant after three centrifugation cycles

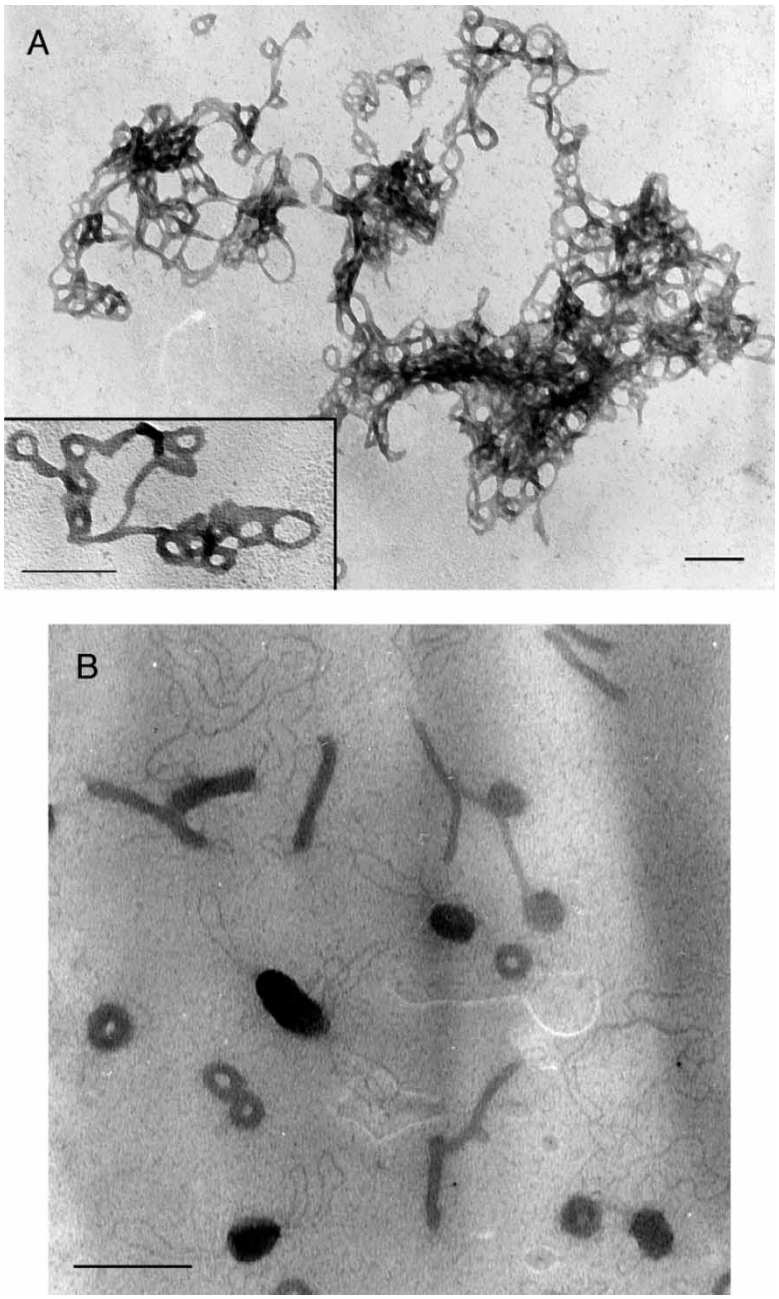

FIGURE 1 Transmission electron micrograph (TEM) of polyplexes (50 μg of pCMVLuc; N/P = 5) prepared with 25 kDa B-PEI-PEG_{3400}-Gal 4% conjugates following the PEI pre-grafting strategy (A) and post-grafted 22 kDa L-PEI/DNA polyplexes (50 μg of pCMVLuc in 1 ml of a 5% glucose solution; N/P = 5) with 2.5% Gal-PEG_{3400}-SC derivative (B). Bars indicate 100 nm.

(500*g*, 10 min) and could be recovered (Fig. 6A and B). Low amounts ($\leq$20% at N/P $\geq$8) of particles stuck to the filter, and some radioactivity, that may be attributed to traces of ^{32}P-labeled nucleotides coming from the labeling procedure, went through the filter. We also verified that both forms of PEI entirely passed through the filter, using fluorescent polycations and that naked DNA was totally recovered in the supernatant (Fig. 6C). After ultrafiltration, the free polymer was recovered in the filtrate (Fig. 6D). As expected, the amount of free PEI increased with the N/P ratio used to prepare complexes and reached values of about 50% at N/P = 10 for both PEIs. Determination of DNA amounts recovered after the ultra-filtration showed that more than 70% of total plasmid DNA was present and that only a low quantity of DNA precipitated on the filter.

Physical Characteristics of the Purified Polyplexes

Particle size measurements done by DLS (Fig. 7) showed that the purification protocol at worst increased the particle size by 10–30 nm. Moreover, the solutions remained mono-disperse and stable over several days. At the end of each cycle of centrifugation, the volume of the DNA-containing solution was reduced by 10. So this purification procedure represents also a mean to quickly prepare

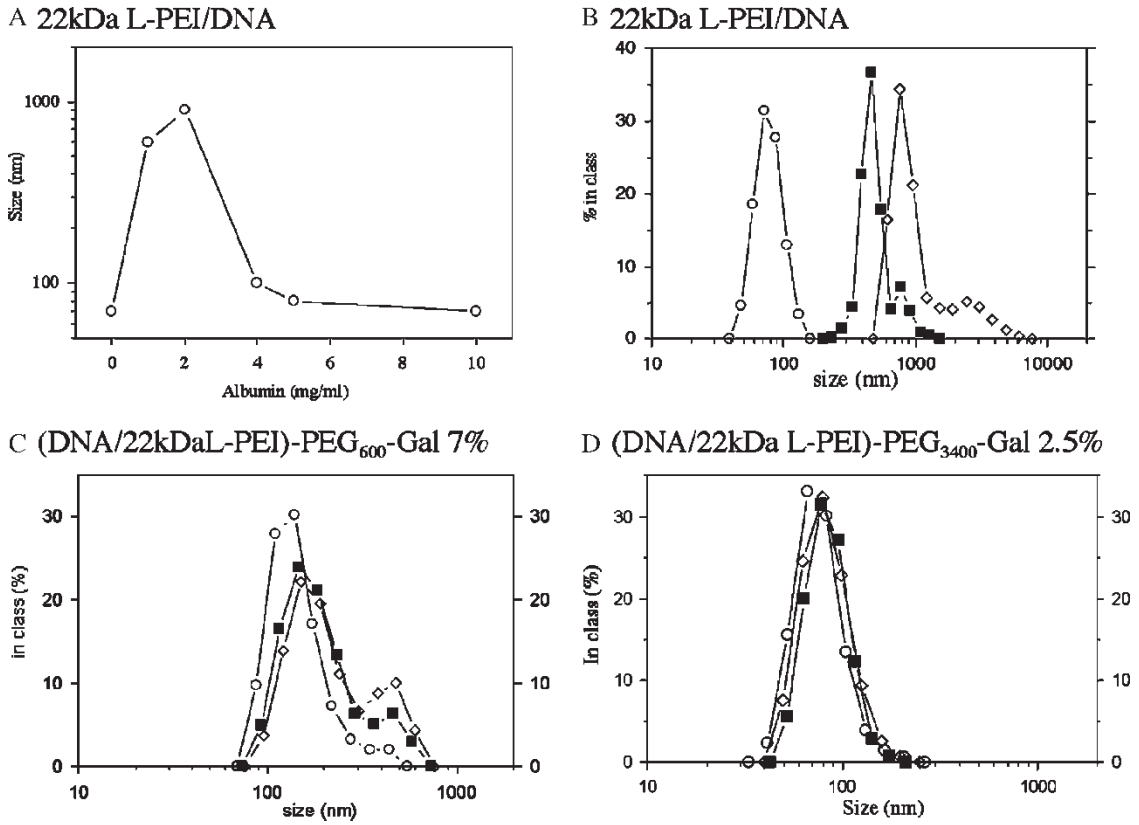

FIGURE 2 Behaviour of unpurified 22 kDa L-PEI/DNA polyplexes and post-grafted polyplexes, in the presence of albumin and under physiological saline concentration. (A) Mean size of polyplexes as a function of albumin concentration. (B–D) Polyplexes were prepared at N/P = 5 and 20 μg/ml DNA, either in water (circle), water + 1 mg/ml albumin (lozenge) or 150 mM NaCl (filled square). The polyplex sizes were determined by dynamic light scattering after 30 min of incubation.

stable, small and highly concentrated DNA-containing particles (up to 4 mg/ml DNA). The size range of the purified particles was confirmed by electron microscopy (Fig. 8).

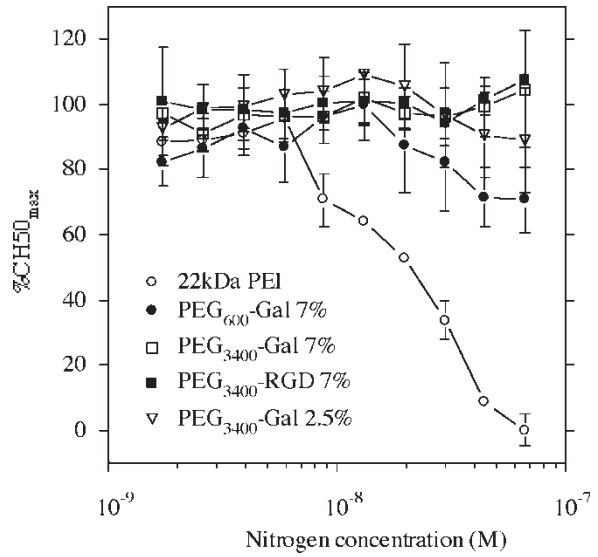

FIGURE 3 Activation of the complement system as a function of polymer amine concentration. Low values represent high activation, see Plank *et al.*, (1996) for experimental conditions.

Cytotoxicity and Transfection Efficiency of Pure Polyplexes

The *in vitro* polymer-associated toxicity in the absence of DNA was determined using the MTT assay (Fig. 9). For N/P ratios 1–20 (6–120μM), B-PEI showed low toxicity (viability >80%). Depending on the cell line, L-PEI appeared more toxic. On HeLa cells, less than 50% of the cells remained viable 24 h after incubation with L-PEI at N/P = 20. For B-PEI, the LD_{50} was reached for 300 (N/P = 50; 13 μg/ml), 600 and 900 μM polymer on HeLa, 3T3, and BNL.CL2 cells, respectively. For L-PEI, LD_{50} was reached for 90, 300 and 300 μM on HeLa, 3T3, and BNL.CL2 cells, respectively.

Using 2 μg DNA for transfection (Fig. 10A–D), luciferase gene expression showed a slight increase with genuine PEI/DNA complexes vs. the crude ones. This effect may be related to the slight viability increase obtained with purified complexes (especially for L-PEI). Increasing the DNA dose used for transfection enhanced the differences between pure and crude complexes (Fig. 10E–H). A strong toxicity was observed using 10 μg DNA with the crude complexes whereas cell viability was unchanged with pure particles.

To confirm the deleterious effect of free PEI, pure polyplexes were re-mixed with some free polymer and added to the cells (Fig. 11). Transfection efficiency and

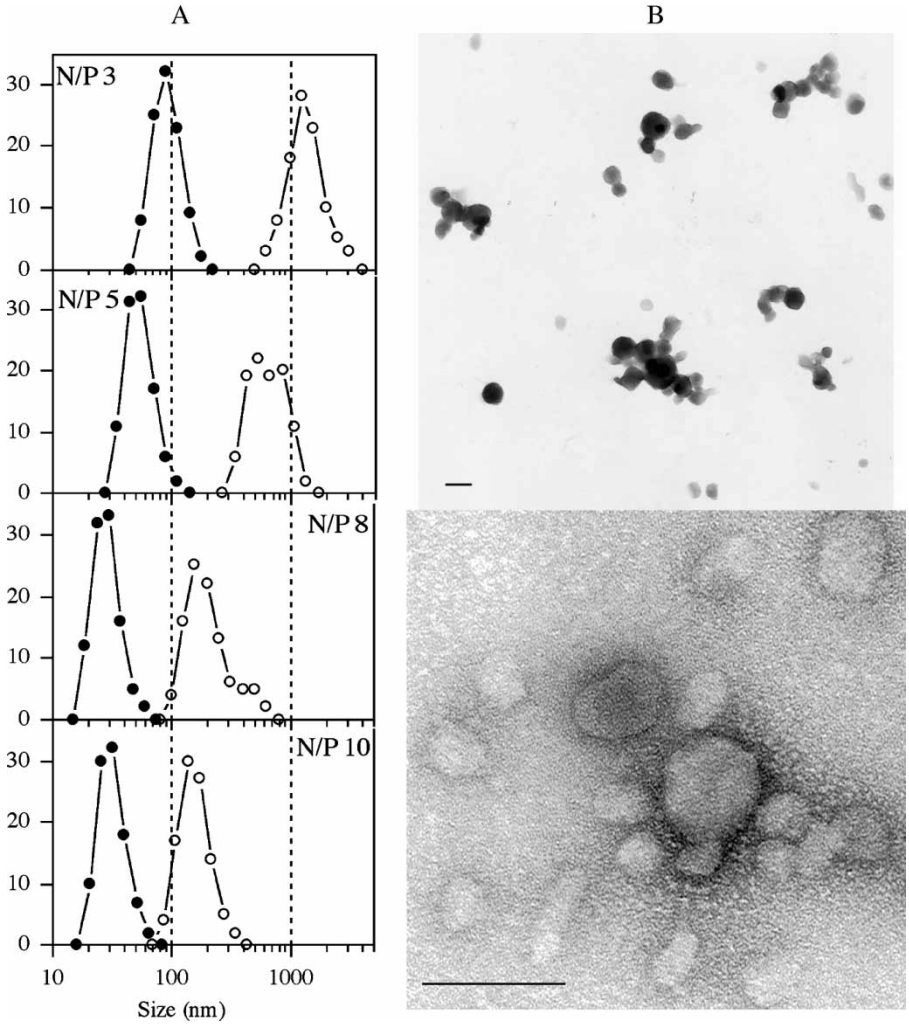

FIGURE 4 B-PEI/DNA complexes (25 kDa) have different sizes according to the formulation conditions. Complexes were prepared in 20 mM HEPES pH 7.4 (A) filled circles; (B) lower panel, or in 150 mM NaCl solution (A) open circles; (B) upper panel. (A) Thirty minutes after complex formation, the particles sizes were determined by dynamic light scattering. (B) Transmission electron microscopy. Bars indicate 100 nm.

the cell survival markedly decreased with increasing amounts of free PEI.

Gene Delivery with the Pure Complexes Following Intravenous Injection

It was shown that L-PEI confers a lung-expression tropism to plasmid DNA when injected into the mouse tail vein in a 5% glucose solution (Goula *et al.*, 1998a; Zou *et al.*, 2000). Optimal transfection conditions were obtained by injecting complexes prepared at $N/P = 10$, hence containing a large excess of PEI (60%). A 40- to 50-fold decrease in gene expression in the lung was observed for $N/P = 4$, i.e. when most of the polymer is associated with DNA. We, therefore, decided to inject the purified complexes containing 50 μg DNA at an initial ratio of $N/P = 10$. A 6-fold decrease of gene expression was obtained in the lungs compared to the unpurified complexes. Increasing the DNA dose to 100 μg reduced the difference of efficiency between pure and crude particles. At 150 μg DNA, only pure complexes allowed gene expression whereas the mice died within hours with the crude complexes.

Influence of the Purification of Polyplexes on PEG Grafting Yields

Table I shows the grafting extent, as percentage of the total amine content (i.e. amino groups of PEI), after a coupling reaction between a reactive PEG and polyplexes, in three situations: pre- and post-grafting using the crude polyplexes, as well as post-grafting using the pure polyplexes. For comparative purposes, the amounts of PEI, DNA and PEG were the same. After 24 h, the non-reacted PEG was removed by extensive dialysis against water. The molecular weight cut-off of the membrane did not allow free PEG–PEI or PEI molecules to diffuse out. The final reaction yield was determined as described in the "Material and Methods Section", and showed a clear dependency on the presence of free polymer molecules, as is dropped from 3.9 down to 1.5%.

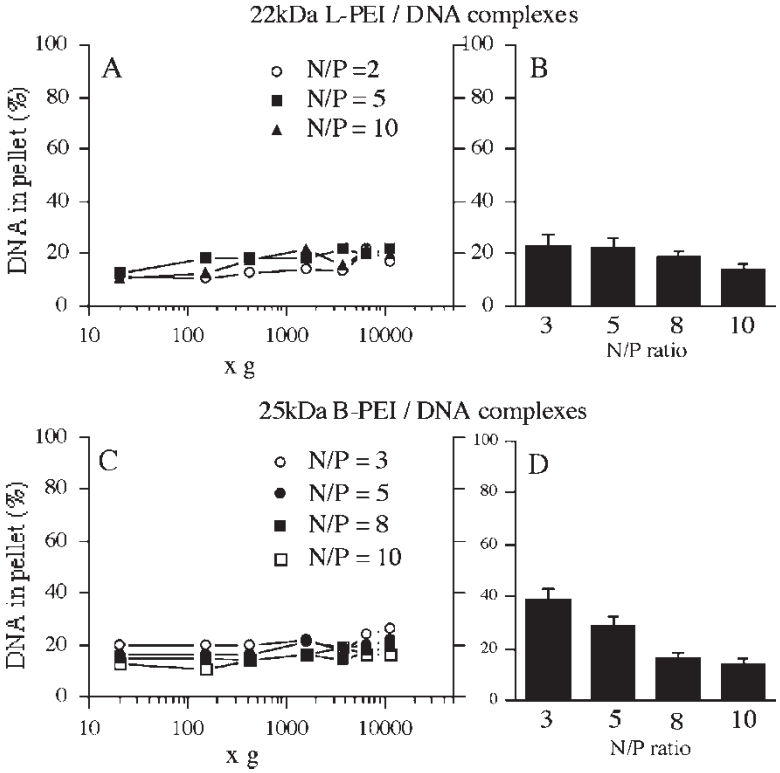

FIGURE 5 Sedimentation of radio-labeled polyplexes in microcentrifuge tubes. (A) Single centrifugation of 10 min. (B) Three cycles of centrifugation at 500*g* for 10 min each.

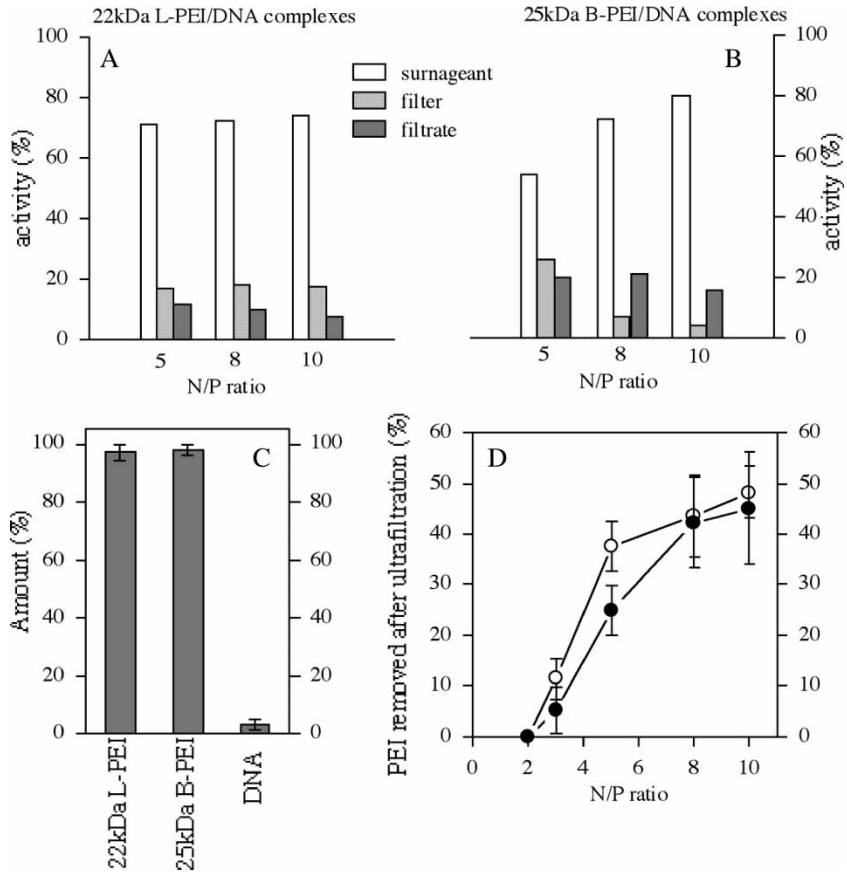

FIGURE 6 Purification of PEI/DNA polyplexes, ultra-filtered 3 times at 500*g* for 10 min. (A and B) PEI/^{32}P-labeled polyplexes. Supernatants, filters, and filtrates (pooled) were counted separately. (C) Percentage of FITC-labeled PEIs (fluorescence; 1.5 μmol corresponding to N/P5 with 100 μg DNA) or free DNA ($OD_{260\,nm}$; 100 μg pCMV-Luc) passing through the filter unit. (D) Percentage of the PEI dose removed after filtration, as a function of the starting N/P ratio. The amount of PEI that passed through the filter was measured by standard ninhydrin and TNBS assays.

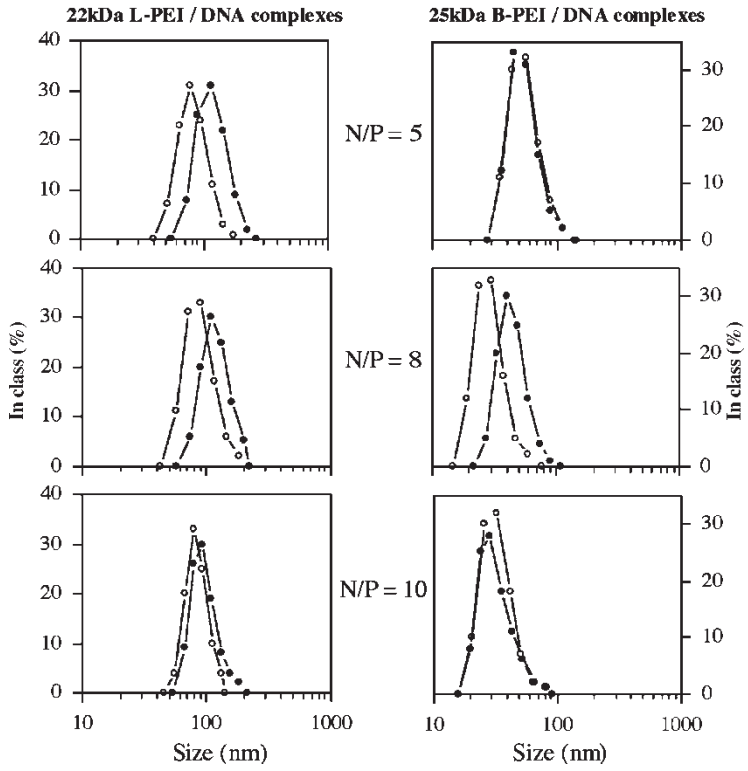

FIGURE 7 Dynamic light scattering size determination of polyplexes before (open circles) and after (closed circles) purification.

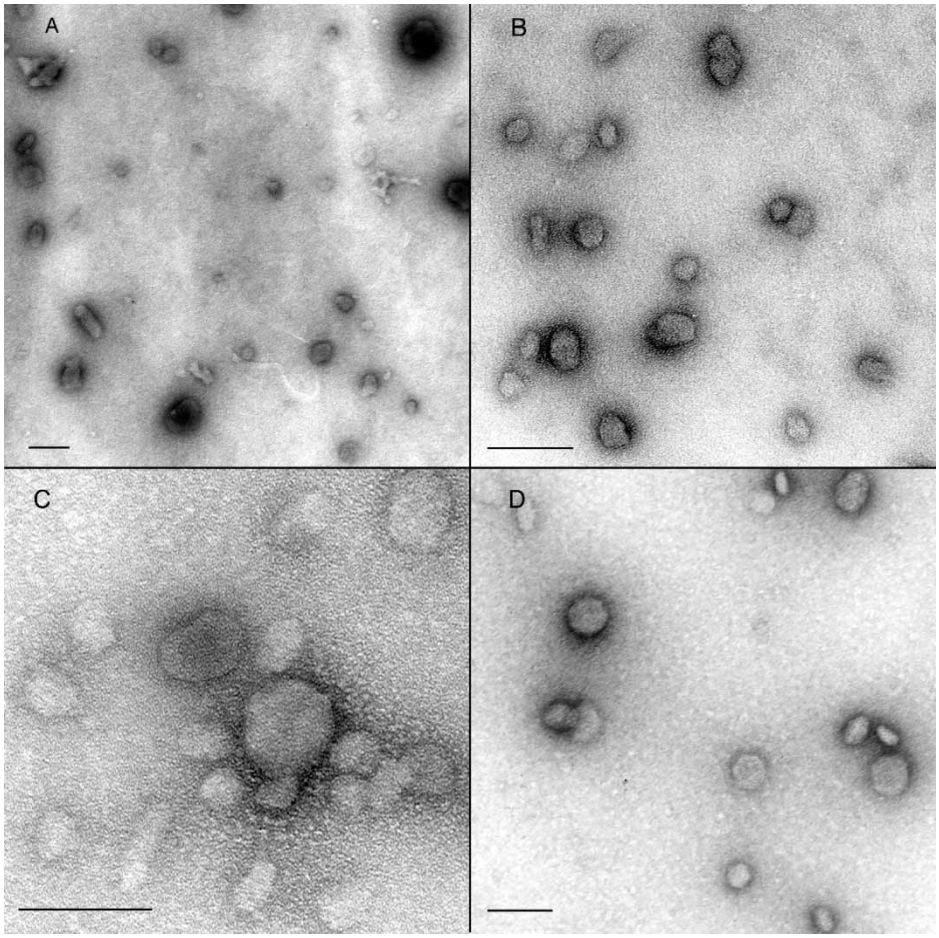

FIGURE 8 TEM of 22 kDa L-PEI (A, C) and 25 kDa (B, D) polyplexes (N/P = 10), before (A, B) and after (C, D) purification.

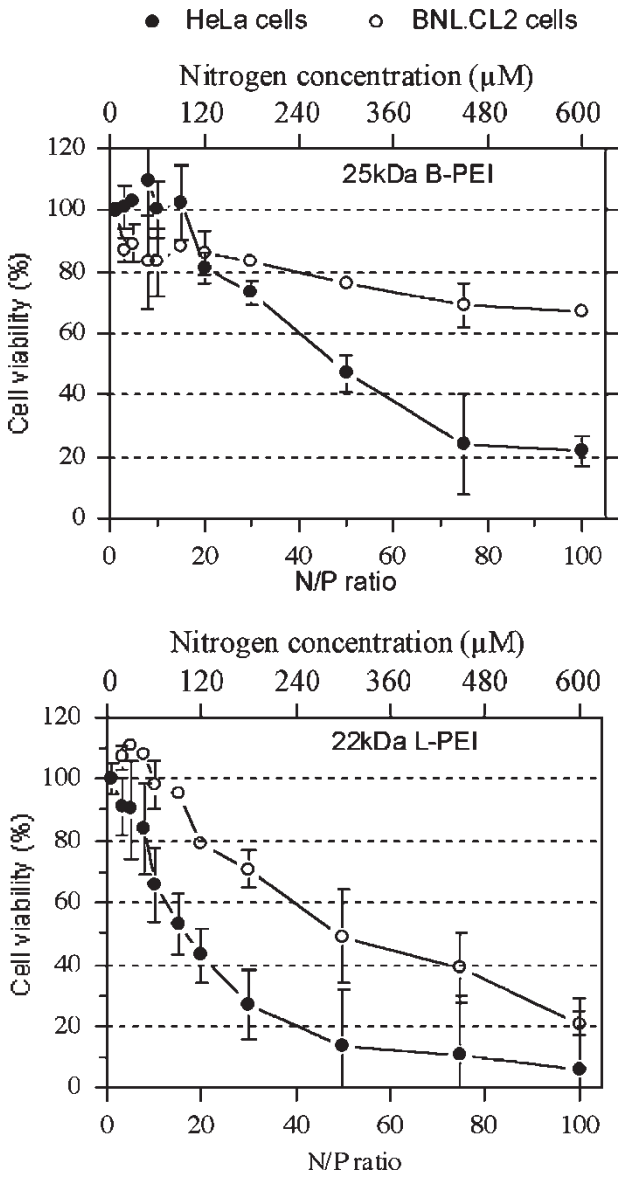

FIGURE 9 Cell viability as a function of free PEI dose (expressed as "pseudo N/P ratio with 2 μg DNA" and μg/ml). Cells were cultured in 24-well tissue culture plate in 1 ml of 10% serum-containing medium and cell viability was measured after 24 h by the MTT assay ($n = 4$, ± S.D.).

DISCUSSION

Systemic delivery of DNA vectors has received increasing interest, as diffusion of particles within tissues was recognized as being a physical barrier. However, results obtained so far for cationic polymer/DNA complexes were disappointing (Wolfert *et al.*, 1996; Ogris *et al.*, 1999; Nguyen *et al.*, 2000; Benns *et al.*, 2001; Blessing *et al.*, 2001). In fact, biological properties of polyplexes are strongly related to their physico-chemical properties, namely their size, morphology, surface charge and colloidal stability (Tang and Szoka, 1997; Ogris *et al.*, 1998; Erbacher *et al.*, 1999a; Wightman *et al.*, 2001). Over the years, we succeeded in preparing small L-PEI/DNA particles that were effective *in vivo* (Goula *et al.*, 1998b). However, their lung-expression tropism, being due to a bolus effect (Zou *et al.*, 2000), the need for vectors that could form "stealth" complexes with DNA, in order to prevent their clearance by the reticulo-endothelial system, became obvious. This has already been achieved in the nanoparticle and liposome fields, by grafting polyethylene (glycol) onto the surface of the vehicles (Allen *et al.*, 1991; Papahadjopoulos *et al.*, 1991; Woodle *et al.*, 1992; Zalipsky *et al.*, 1994). Recent studies have also described PEGylated cationic polymer systems, some of them reporting promising *in vivo* results (Ogris *et al.*, 1999; Kircheis *et al.*, 2001). However, the chemistries used and the lack of purification led to rather loosely characterized delivery systems.

Pre-grafted PEG–PEI/DNA polyplexes displayed very attractive physical characteristics, as light scattering and zeta potential measurements gave values corresponding to small (30 nm) and neutral particles (Erbacher *et al.*, 1999a). Electron micrographs were in contradiction with the indirect sizing technique. In fact, random grafting of 2–10 PEG_{3400} molecules on each PEI polymer seems to interfere with its DNA condensing ability. Assuming compact spheroidal particles, the odd scattered light data were converted by the instrument into small "particles". These observations have been also described by Rackstraw *et al.* (2001).

We decided to circumvent this interference by directly attaching the PEG moiety to pre-formed polyplexes. Amino-hydroxy-PEG3400 was converted to pyridyldithio-hydroxy-PEG by reaction with the commercially available SPDP reagent. It was further activated as a succinimidyl carbonate on its hydroxy terminus by reacting it with the commercially available DSC reagent. The final hetero-bifunctional PEG derivative was added to the pre-formed polyplexes. Reaction between amino groups of the PEI and functionalized PEG led to pyridyldithio-PEG coated particles. Thio-galactose or a cystein-containing RGD peptide (Erbacher *et al.*, 1999b) were further coupled the distal end, yielding the ligand–PEG-particles. This three-step protocol allowed the formation of post-grafted PEGylated and cell-targeting polyplexes. In order to obtain formulations that are consistent with the biophysical characteristics required for systemic delivery, we needed to start with small PEI/DNA complexes. L-PEI was already shown to form small particles with DNA in 5% glucose solution. As we found out, B-PEI can form even smaller particles with DNA if complexes are prepared in low ionic strength Hepes buffer, at physiological pH. Both L-PEI- and B-PEI-based particles were post-grafted with PEG. The resulting complexes displayed no longer "hair-like" structures, but rather spherical, toroidal or rod-like ones. These structures were already observed with other vectors, such as chitosan (Erbacher *et al.*, 1998), and were shown to be compatible with gene delivery. In most cases however, some 5–10 nm-thick strains were seen looping out of the complexes.

The resistance to salt- and protein-induced aggregation and to complement activation are challenges in the formulation of vectors displaying the desired steric stability

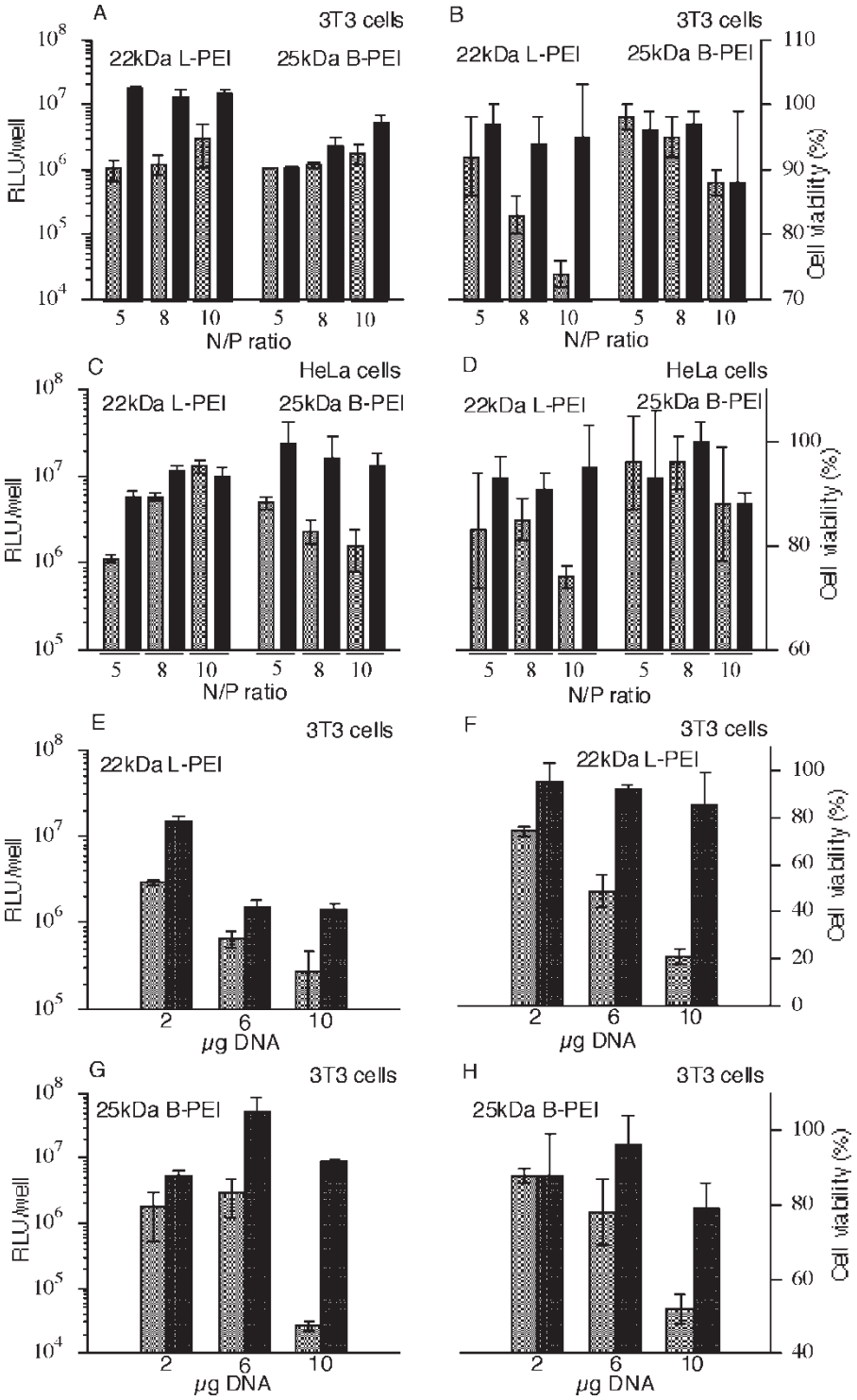

FIGURE 10 Transfection efficiency and cell viability of purified (filled bars) and unpurified (hatched bars) polyplexes, as a function of N/P ratio with 2 μg/well DNA (A–D) or DNA dose at N/P = 10 (E–H). Transfections (24-well tissue culture plate; 1 ml of 10% serum-containing medium) were performed for 24 h with pCMV-Luc plasmid and gene activity was expressed as relative light units integrated over 10 s, per well, ± S.D. ($n = 3$). Cell viability was measured after 24 h by the MTT assay ($n = 3$, ± S.D.).

and inertness under physiological conditions (Finsinger *et al.*, 2000). The chosen post-grafting strategy proved successful in that PEG of high molecular weight was able to protect polyplexes under conditions mimicking systemic delivery. Together with higher *in vitro* transfection efficiencies, as compared to the pre-grafted particles, these results were encouraging. However, PEG post-grafted polyplexes proved to be unable to deliver genes *in vivo*, whether targeting cells or not (RGD–PEG–PEI/pCMV–Luc injected in tumor-bearing mice, JLC unpublished data). Moreover, the *in vitro* transfection levels were neither depending on the presence, nor on

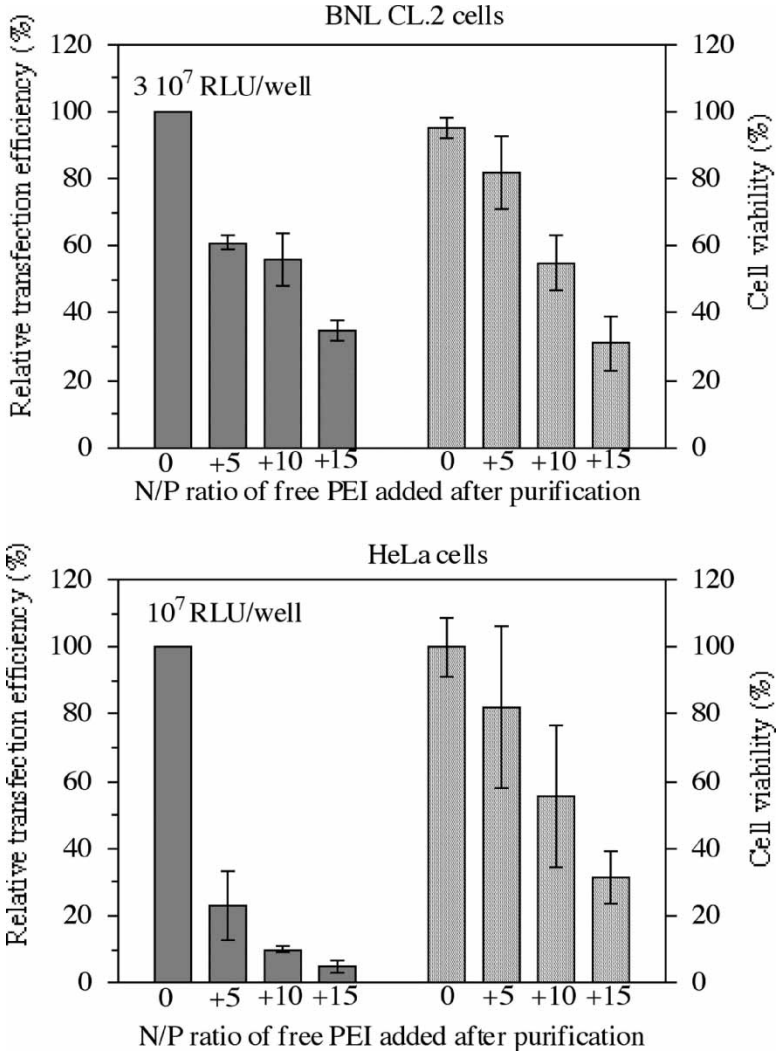

FIGURE 11 Transfection efficacy and cell viability as a function of free 22 kDa L-PEI. Polyplexes were prepared at an initial N/P = 10 and subjected to purification to obtain a final N/P = 5. Purified polyplexes corresponding to 2 μg DNA were added in each well (triplicates), together with various amounts of free PEI (expressed as N/P ratios equivalent to polyplexes prepared with 2 μg DNA). added to 22 kDa L-PEI/DNA purified complexes (initial N/P = 10). Added free PEI is expressed as "pseudo equivalents". Transfection efficiency and cell viability are expressed as percentage of the maximal value (no free PEI added). Mean ± S.D. are given ($n = 3$).

the nature of the ligands, irrespective of the transfected cell type (TB and PE, unpublished results).

This strongly suggested that the amount of PEI used for vector formulation was not entirely incorporated into vector particles and remained either free, or loosely bound to particles. This excess PEI would be disadvantageous in a post-grafting strategy and may even prove inhibitory in a combined post-grafting and targeting approach. Therefore, we went back to unmodified PEIs, and developed a protocol to remove potentially "free" cationic polymers. Ultra-filtration through molecular weight cut-off membrane embedded into centrifuged device for centrifugation-accelerated filtration seemed to be the most convenient technique. We first verified that small polyplexes did not sediment under the chosen conditions. In fact, at any speed up to 10,000 *g*, not more than 20% of the particles were sedimented, in a microcentrifuge tube assay. We also verified that polyplexes were behaving properly under the conditions used for purification (3 cycles of 10 min at 500 *g*; Figs. 5B,D, and 6A,B), and that free PEI molecules were passing through the filter membrane, in contrast to plasmid DNA (Fig. 6C). We then ran the purification protocol on polyplexes. The proportion of free PEI depended on the initial N/P ratio and on the PEI structure (linear vs. branched; Fig. 6D). The data can be interpreted such that roughly up to 2–3

TABLE I

Conditions*	PEG grafting extent (%)
22 kDa L-PEI	11
Crude 22 kDa L-PEI/DNA complexes (N/P = 8)	3.9
Pure 22 kDa L-PEI/DNA complexes (initial N/P = 8)	1.5

* PEI nitrogen (3 μmol); pCMVLuc plasmid at 100 μg/ml, reaction with 1.5 μmol of Py-SS-PEG_{3400}-SC in 1 ml of water for 24 h.

L-PEI and 4–5 B-PEI equivalents (in terms of the N/P ratio) can tightly bind to DNA and stay in the core of the complex, whatever the initial PEI input was; above this, the remaining polymer being free in solution. However, the initial high input of polymer is vital for the formation of small polyplexes. Interestingly, once the complexes are formed at a high input ratio, the removal of free PEI does not alter the physical characteristics of L- and B-PEI/DNA particles (Figs. 7 and 8), except the zeta potentials that were slightly lower (ca. 10 mV; data not shown) at their cationic plateau. It is also remarkable that during the purification protocol, particles were concentrated up to 4 mg DNA per ml, a fact that might be of interest for some *in vivo* transfection protocols.

PEI, just like other cationic polymers, is toxic to cells, when added as a free molecule. The LD_{50}'s depend on the cell type and polymer structure. However, the polymer concentrations that are toxic are much higher than those commonly used in *in vitro* gene delivery (Fig. 9), except for 22 kDa L-PEI on HeLa cells. Yet, this does not rule out a synergistic toxicity effect between polyplexes and free polymer molecules. In fact, this is exactly what is observed *in vitro* (Fig. 10). Comparing pure and crude complexes as a function of N/P ratio clearly shows that pure particles exhibit transfection efficacies ranging from similar to 10-fold better. Along the same line, cell viability is sometimes increased when pure particles were used. Using escalating doses of polyplexes further supported this finding: pure particles are less toxic and more efficient than crude ones, the later effect being possibly a consequence of the former. This was confirmed by adding back free polymers to the pure complexes, just before transfecting cells. The results shown in Fig. 11 undoubtedly confirm the deleterious effect of free polymer on both cell viability and transgene expression levels.

In a preliminary set of *in vivo* experiments, we injected mice in the tail vein with polyplexes prepared at N/P = 10, in 3 escalating doses (50, 100 and 150 μg DNA), with either pure or crude non-grafted polyplexes. Although it is too early to draw final conclusions, there was a clearly toxic effect of crude complexes at the highest dose, that was not observed with the pure complexes.

Coming back to the preparation of "stealth" polyplexes for *in vivo* experiments, we compared the extent of PEG grafting on three types of formulations (Table I). The pre-grafting strategy is a "regular" chemical reaction, in which free molecules are coupled together. We obtained 11% of PEG grafting to PEI. Quantitative reaction would have led to 50% grafting, i.e. the chemical coupling yield was ca. 20%. Post-grafting the pure pre-formed polyplexes gave 1.5% grafting, which represents a chemical yield of 3%. It is therefore clear that PEGylation occurs preferentially (20:3) on free polymer, instead on the particles surface. As a consequence, published *in vivo* data using crude complexes may not be representative of the properties of pure cationic polymer/DNA complexes.

MATERIALS AND METHODS

Chemicals

Branched PEI (B-PEI, average MW = 25 kDa, average degree of polymerization = 580) and linear PEI (L-PEI, average MW = 22 kDa, average degree of polymerization = 510, Exgen 500) were obtained from Aldrich (St. Quentin Fallavier, France) and Euromedex (Souffelweyersheim, France), respectively.

Particle Size and Zeta Potential Measurements

B-PEI-based complexes were prepared in 20 mM HEPES buffer, pH 7.4 and L-PEI-based complexes in a 5% glucose solution. To 100 μg pCMVLuc plasmid in 500 μl solution, the desired amount of PEI in 500 μl solution was added, mixed, and left for 15 min at room temperature. Particle size or zeta potential was measured using a Zetasizer 3000 (Malvern Instrument, Orsay, France) with specifications as described previously (Erbacher *et al.*, 1999a). DNA-containing complexes were also observed by transmission electron microscopy (TEM), with a Philips EM 410 transmission electron microscope, as described previously (Erbacher *et al.*, 1999a).

Synthesis of PEI–PEG-SS-Galactose

$HCl{\cdot}H_2N{-}PEG_{3400}{-}COOH$ (Shearwater Corporation, Huntsville, AL) was first modified to a hetero-bifunctional HOOC–PEG–SS–Pyridine derivative before grafting to PEI as previously described (Erbacher *et al.*, 1999a). The resulting PEI–PEG–SS–Pyridine conjugate was converted to PEI–PEG–SS–Galactose by addition of 1-thio-β-D-Galactopyranose (Sigma, St. Quentin Fallavier, France) as described by Erbacher *et al.* (1999a).

Post-grafting of PEI/DNA Complexes with PEG–ligand Conjugates

L-PEI/DNA complexes (22 kDa) were prepared in 1 ml water (100 μg of pCMVLuc; N/P = 5–8). A measured quantity of 0.6–3 μmol of heterobifunctional α-pyridyl-dithio-ω-hydroxy-PEG-succinimidyl carbonate derivative (Py-SS-$PEG_{600\text{ or }3400}$-SC, synthesis described in Bettinger *et al.* (1998)) were added and the solution was left 12 h at room temperature. Excess PEG derivatives were removed by dialysis against water for two days (MWCO 100,000; Dispodialyser Spectra/Por®). The post-grafting yield was estimated by release of 2-thiopyridone following reduction with excess DTT (monitoring at 343 nm). Two PEG_{3400}SS-Py-post-grafted complexes were prepared, having 2.5 and 7% of derivatized amine functions. One PEG_{600}SS-Py-post-grafted complexes was prepared, having 7% of derivatized amine functions. The final targeted complexes were obtained after addition of two molar equivalents of

1-thio-β-D-Galactopyranose or HS-CYGGRGDTP peptide (Genosys, Cambridge, UK). The reaction was completed within 30 min, as indicated by the formation of 2-thiopyridone (about 95% yield). Excess of low molecular weight ligands were removed by dialysis against water for two days (MWCO 10,000, Spectra/por).

DNA Labeling Protocol and Sedimentation Experiments

pCMVLuc plamid (25 ng) was radiolabeled by random priming with a Megaprime DNA labeling kit (Amersham, Les Ulis, France) and [α-^{32}P] dCTP (3000 Ci/mmol, Amersham) yielding to 2.4×10^9 cpm/μg DNA. The DNA probe was then added to 250 μg unlabeled plasmid giving a final activity of 9.6×10^6 cpm/μg DNA. To prepare complexes, 2 μg pCMVLuc and various amounts of polycation were diluted separately in 100 μl 2 mM HEPES pH7.4 (B-PEI), and in a 5% glucose solution (L-PEI), in prelubricated microcentrifuge tubes (Costar, Cambridge, MA). After 10 min, the vector was added to the DNA; the resulting solution was homogenized and left for 15 min at room temperature. Complexes were then centrifuged at various speed (17, 150, 420, 1620, 3770, 6700, 11340*g*) for 10 min. Supernatants (180 μl) and pellets (20 μl) were counted separately in a scintillation counter, using the Cerenkov procedure. The percentage of sedimented DNA/polymer complexes was calculated according to the following formula:

$$\left[(\mathrm{CPM_{pellet}} - 0.1 \times \mathrm{CPM_{supernatant}})/ (\mathrm{CPM_{pellet}} + \mathrm{CPM_{supernatant}})\right] \times 100.$$

Purification of DNA Complexes by Ultrafiltration

Polyplexes (100 μg DNA per ml in 20 mM HEPES pH7.4 or 5% glucose solution) were centrifuged through Centricon-100 membranes (Millipore, Saint-Quentin, France) 3 times 10 min at 500*g* on a Sigma K10 fixed angle rotor. Between centrifugations the polyplex solutions were rediluted to their original concentrations.

In order to measure the fraction of PEI present in the PEI/DNA complexes, PEI was labelled with fluorescein isothiocyanate (Sigma) in a 0.2 M borate buffer pH 8.4. The conjugate was purified by gel filtration on a PD10 column (Sephadex G-25 M, Pharmacie Biotech, Sweden) in water. The concentration of PEI amine functions (N) was determined with the ninhydrin assay. The level of fluorescein conjugation per amino group of PEI and was 1% as measured by absorption spectroscopy at 495 nm. After the polyplex centrifugation step through Centricon-100 membranes, the amount of free PEI-fluorescein present in the filtrate was determined by absorbance at 495 nm, from which the fraction of PEI that was associated with DNA was calculated.

In Vitro Transfection

HeLa human cervix epitheloid carcinoma (kindly given by Dr L. Monaco, San Raffaele Scientific Institute, Milan, Italy) were grown in Minimum Essential Medium (MEM) with Earle's salt (PolyLabo, Strasbourg, France) and supplemented with 10% fetal calf serum (FCS, D. Dutcher, Brumath, France), 2 mM glutamine (Gibco BRL), 100 units/ml penicillin (Gibco BRL) and 100 μg/ml streptomycin (Gibco BRL). Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. The desired amount of polycation and 6 μg of pCMVLuc (for a triplicate experiment) were diluted separately in 150 μl of 20 mM Hepes buffer (B-PEI), pH7.4 or in a 5% glucose solution (L-PEI). After 10 min, the polycation was added to the DNA solution; the mixture was homogenized and left for 10 min. Twenty four hours before transfection, $5-6 \times 10^4$cells/well were seeded in 24-well tissue culture plates. Before transfection, cells were supplemented with 1 ml fresh complete medium containing 10% FCS. Then, 100 μl of polycation/DNA complexes were added per well and the plates were incubated at 37°C for 24 h. When specified, HeLa cells were transfected in the absence of serum. In this case, 10% serum was added to the cells after 2 h of incubation. Luciferase gene expression was measured using a commercial kit (Promega, Cergy Pontoise, France) and a luminometer (Mediators PhL, Vienna, Austria). Results were expressed as light units integrated over 10 s, per mg of cell protein using the BCA assay (Pierce, Paris, France). Each experiment was done in triplicate.

Intravenous Injection of L-PEI/DNA Complexes in the Mouse Tail Vein

Female Swiss nude mice (4 weeks old, IFFA-CREDO, Marcy l'Etoile, France) were placed in a restrainer and 200 μl of unpurified or purified 22 kDa L-PEI/DNA complexes/mouse ($n = 3$), prepared in a 5% glucose solution at a N/P = 10, were injected rapidly (5 s) in the tail vein, using a 1/2 in 26-gauge needle and a 1 ml syringe (Fig. 12). Animals were killed by cervical dislocation and lungs were collected, washed with PBS, homogenized, and transferred in 1.5 ml microcentrifuge tubes containing 1 ml pre-cooled lysis buffer (Promega). Homogenized tissues were subjected to a freeze cycle (−20°C) for one day. The homogenates were thawed, left at room temperature for 30 min and then centrifuged at 14,000*g* for 5 min. Ten microliters supernatant aliquots were assayed for luciferase activity using a commercial kit (Promega) and a luminometer (Berthold, Paris, France). Results were expressed as light units integrated over 10 s, per mg of cell protein using the BCA assay (RLU/mg of protein; 2 μg of purified firefly luciferase produced 10^8 RLU/10 s).

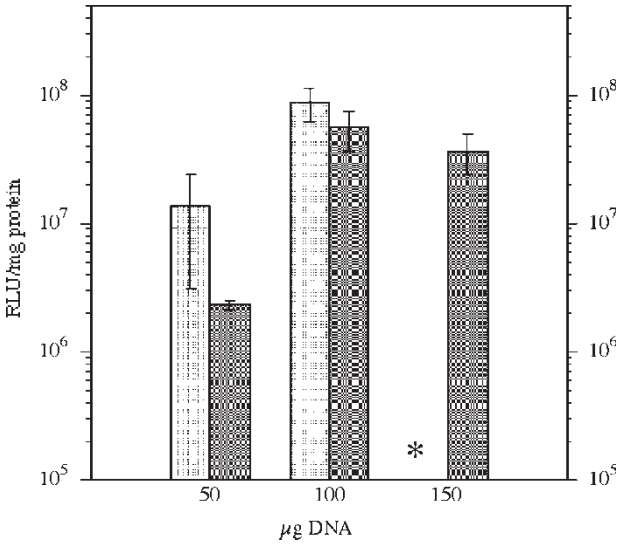

FIGURE 12 *In vivo* luciferase expression in Swiss nude mouse lungs after tail vein injection of 22 kDa L-PEI/DNA polyplexes, either unpurified (light gray bars) or purified (dark gray bars), at N/P = 10. Mean ± S.D. are given ($n = 3$). * represents 100% lethality (after 30 min).

Acknowledgements

T.B. and P.E. was recipient of a doctoral fellowship from the 'Ministère de l'Enseignement supérieur, de la Recherche et des Technologies' and a post-doctoral fellowship from 'Sidaction', respectively. This work was supported by the ARC (Association pour la Recherche contre le Cancer).

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