Cellular Delivery and Antisense Effects of Peptide Nucleic Acid Conjugated to Polyethyleneimine via Disulfide Linkers

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Peptide nucleic acid (PNA) is potentially an attractive antisense and antigene agent for which more efficient cellular delivery systems are still warranted. The cationic polymer polyethylenimine (PEI) is commonly used for cellular transfection of DNA and RNA complexes, but is not readily applicable for PNA due to the (inherent) charge neutrality of PNA. However, PEI could function as an efficient scaffold for PNA via chemical conjugation. Accordingly, we modified PEI with the amine-reactive heterobifunctional linker agent *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (with and without a PEG moiety) and further reacted this with a cysteine PNA. The level of modification was determined spectrophotometrically with high accuracy, and the PNA transfection efficiency of the conjugates was evaluated in an antisense luciferase splice-correction assay using HeLa pLuc705 cells. We find that PEI is an efficient vector for PNA delivery yielding significantly higher (up to 10-fold) antisense activity than an analogous PNA−octaarginine conjugate, even in the presence of chloroquine, which only slightly enhances the PEI-PNA activity. The PEI-PEG conjugates are preferred due to lower acute cellular toxicity. Finally, the method can be easily modified to allow for co-conjugation of other small molecules in a high-throughput screening assay that does not require a purification step.

INTRODUCTION

Peptide nucleic acids (PNAs[1]) have many of the properties desired of a gene targeting and antisense agent (*1*−*3*), but efficient cellular delivery and in vivo bioavailability are still a challenge despite the development of a variety of delivery techniques, including the use of cationic cell penetrating peptides (*4*−*7*) and cationic lipid transfection (*8*−*11*).

Polyethylenimine (PEI) is widely used as an efficient, nonviral complexation transfection agent for RNA and plasmid DNA (*12, 13*), and it is commercially available in average polymer sizes ranging from ∼0.5 to 800 kDa (*14*). The high transfection efficiency of PEI is considered to arise from what has been coined the "proton sponge" effect (*12, 15*); the high amine content and buffering capacity of PEI results in endosomal import of protons, high osmotic pressure, and subsequent rupture of the endosomes. The endocytotic pathway is not yet clear but has been suggested to be caveolar, since modified PEI directed to clathrin and caveolar pathways is only effective in the latter (*16*). Furthermore, the endocytotic route and endosomal escape may be affected by the DNA/RNA cargo (*13, 17, 18*).

PEI could also be an attractive candidate for PNA delivery. However, because PNA is inherently charge neutral, chemical conjugation rather than simple complexation to PEI is required. The high primary amine content of PEI allows conjugation of a large number of (PNA) ligands using commercially available amine reactive linkers.

PEI transfection efficiency (and toxicity) is mutually correlated to the polymer size and structure, but branched PEI with a molecular weight of 25 kDa (PEI25) is an acceptable compromise between toxicity and transfection efficiency (*19*−*21*). Furthermore, several PEI derivatives with reduced toxicity but retained efficacy have been described, namely, cross-linked PEI (*22*−*24*) or grafted PEI onto less cytotoxic polymers like chitosan (*25*). Thus, we chose PEI25 as a suitable scaffold for PNA conjugation. Finally, modification of PEI with poly(ethylene glycol) (PEG) reduces toxicity of PEI in vitro (*26*−*28*) and in vivo (*29*−*31*), and we therefore decided to also include a PEG containing linker for PNA conjugation.

MATERIALS AND METHODS

Stock solutions of polyethylenimine, M_w 25 kDa (PEI25) and M_w 1.3 kDa (PEI1.3), both from Sigma, were prepared by neutralizing with HCl and accurately diluting to 50 mg/mL.

N-Succinimidyl-3-(2-pyridyldithio)propionate (SPDP) from CalBioChem and SPDP-dPEG$_8$-NHS ester (SPDP-PEG$_8$) from Quanta Biodesign were diluted in dry dimethyl sulfoxide (DMSO, Merck) immediately before use. Buffers used were as follows: 10× phosphate buffered saline (PBS) (100 mM Na_2HPO_4, 20 mM KH_2PO_4, 30 mM KCl, 1.4 M NaCl; all from Merck) adjusted to pH 7.4; HEPES buffer (Sigma), 200 mM, pH 7.4, and borate buffer (0.5 M boric acid (Sigma), pH 9.3). 2,4,6-Trinitrobenzenesulfonic acid (TNBS; Fluka) was used as 0.1 M aliquots, *o*-phthalaldehyde (OPA; Sigma) as 1 M aliquots, and dithiothreitol (DTT; BioUltra, Sigma) as 0.1 M aliquots at pH 9; all were stored at −20 °C.

PNAs were synthesized by standard t-Boc chemistry as previously described (*32, 33*), purified by HPLC, and characterized by MALDI-TOF. Arg$_8$-PNA (PNA2787): H-(D-Arg)$_8$-Gly-CCT CTT ACC TCA GTT ACA-NH_2 (M_w: 6068.5 Da (calc:

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[1] Abbreviations: PNA, peptide nucleic acid; PEI, polyethylenimine; PEI25, branched PEI with M_w 25 kDa; PEG, poly(ethylene glycol); SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; PEI-P, PEI25 with variable ratios of amine linked PDP; PEI-PEGP, same as PEI-P with an 8mer PEG spacer.

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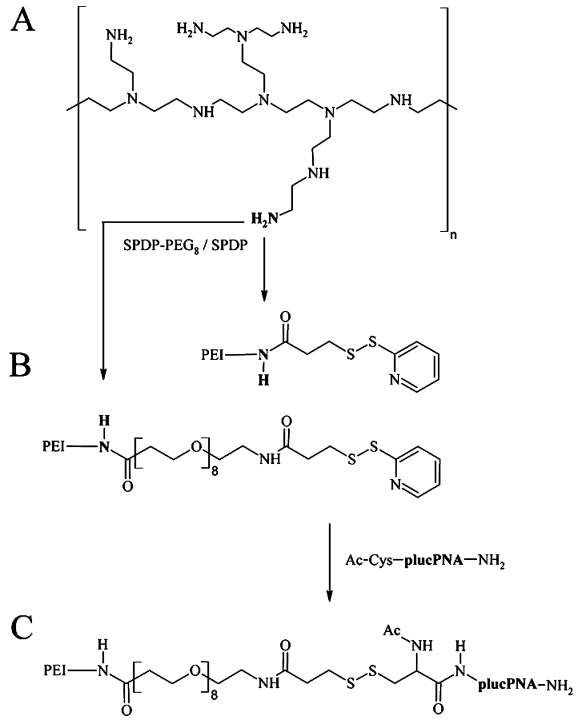

Figure 1. Synthesis and structures of PEI conjugates. Partial structure of polyethylenimine (A) and synthesis strategy. Primary amines were reacted with SPDP or SPDP-PEG_8 reagents containing an *N*-hydroxysuccinimide (NHS) ester and a sulfhydryl-reactive pyridyl disulfide group (B) which could further be reacted with a cysteine-PNA (C). PEI25 was modified to variable degrees with SPDP or SPDP-PEG_8; $n_{average}$ = ca. 15, 30, 68 (see Table 1).

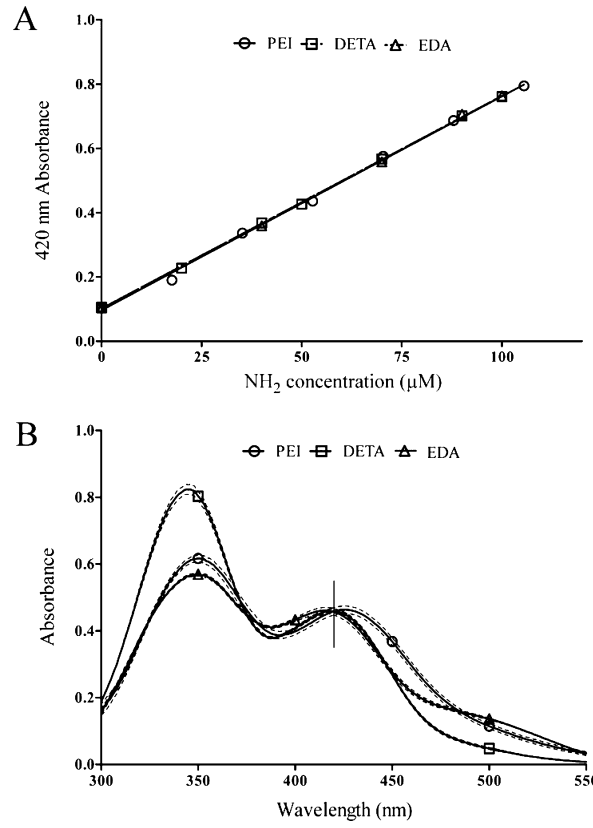

Figure 2. Standard curves (A) and absorption spectra (B) of PEI, DETA, and EDA. Absorption spectra at different concentrations of PEI, DETA, and EDA reacted with TNBS in borate buffer with 3% Tween 20 were recorded after 120 min, and absorbance at 420 nm (A) was plotted against theoretical primary amine concentration (see text for further explanation). TNBS in borate buffer with 3% Tween 20 at 120 min was used as $[NH_2] = 0$. (B) TNBS absorbance was subtracted from raw data and averaged spectra correspond to concentrations of 70 μM NH_2 ($n \geq 4$). Error lines (dashed) = standard deviation.

6071.97). Cys-PNA (PNA3140): Ac-Cys-CCT CTT ACC TCA GTT ACA-NH_2 (M_w: 4916.15 Da (calc. 4910.59). C-teminals were amidated and N-terminal cysteine was acetylated. Cys-PNA solutions were stored at −20 °C to avoid disulfide formation.

Quantification of Polyethylenimine. PEI was quantified from primary amine content using a modified version of the TNBS assay (*34*).

PEI, diethylenetriamine (DETA), and ethylenediamine (EDA) (Sigma) were diluted to appropriate concentration in 0.1 M borate buffer, pH 9.3, containing 3% Tween 20 (Merck) and 1 mM TNBS. Following 120 min reaction, UV−vis absorbance spectra were recorded (Cary 300, Varian Inc.).

Preparation of PDP-Functionalized Polyethylenimine. Five milligrams of PEI25 (0.2 μmol) was dissolved in 1.8 mL 1× PBS, pH 7.4, and SPDP (or SPDP-PEG_8) (5 to 20 μmol in 200 μL dry DMSO) corresponding to a theoretical modification of 25−100 linkers per PEI was added. The reaction was stopped after 8 h by gel filtration using Sephadex G-25 Super Fine (G-25SF, GE Healthcare). The Sephadex was swelled according to manufacturer's protocol and preconditioned overnight with PEI1.3 and PEI25, 1 mg each/g of dry Sephadex. Seven milliliters of swelled gel was packed in handmade columns plugged with glass wool and washed thoroughly with HEPES buffer (20 mM, pH 7.4, filtered and degassed) before applying the sample. Fractions were collected in low-bind 1.5 mL polypropylene tubes using a peristaltic pump at 2.5 mL/min by elution with the HEPES buffer. Fractions containing PEI25 conjugates were pooled and dialyzed (Spectra/Por 2, MWCO 12−14 kDa, Spectrum Laboratories.) for 4 h + 12 h (250× volume, buffer change after 4 h) at 4 °C against ddH_2O. The dialysis membranes were pretreated with OPA in 50% ethanol and DETA to remove unspecified traces of sulfur, and washed thoroughly prior to dialysis.

Transfection Experiments with PEI-P/PEGP. HeLa pLuc705 (Gene Tools LLC) were grown at 37 °C with 5% CO_2 in RPMI1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), 1% GlutaMAX (Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). The day before transfection, cells were seeded in 96-well plates (Nunc), without antibiotics, at 8×10^3 cells per well.

PEI-P/PEGP-PNA conjugates were prepared in 96-well plates; one well was prepared per triplicate transfection. Cys-PNA was left to react with PEI-P/PEGP in 20 mM HEPES in the well for 1 h. At the time of transfection, media was removed and 25 μL of the unpurified PEI-P/PEGP-PNA conjugates were diluted to 100 μL in OPTI-MEM (Gibco), and were transferred to the cells. After 6 h, cells were supplemented with 100 μL of 20% FBS and 1% GlutaMAX in RPMI-1640 and incubated for additional 18 h. For lactate dehydrogenase (LDH) leakage assays (CytoTox-ONE, Promega), 50 μL medium was recovered for analysis. After incubation, the transfection solution was removed, and then 50 μL passive lysis buffer (Promega) was added to the cells, followed by a freeze/thaw cycle, and the lysate was subjected to the further analysis. Ten microliters of lysate was diluted with 40 μL H_2O and analyzed for ATP content (CellTiter-Glo, Promega), while remaining lysate was assayed for luciferase activity (Bright-Glo, Promega). All assays were handled according to manufacturer.

RESULTS AND DISCUSSION

For conjugation to PNA, we chose to focus on PEI25, since it is a well-known standard in transfection with cationic poly-

Table 1. PEI Conjugates Used for Transfection[a]

linker	PEI25 conjugate	PDP/PEI (±SD)
SPDP	PEI-P14	14.4 ± 0.4
	PEI-P29	28.5 ± 0.9
	PEI-P68	68.4 ± 1.9
SPDP-PEG_8	PEI-PEGP15	15.5 ± 0.3
	PEI-PEGP30	29.6 ± 0.4
	PEI-PEGP66	66.1 ± 2.1

[a] Conjugates were characterized by TNBS assay and P2T release by DTT cleavage. SD = standard deviation.

mers and makes a reasonable compromise between transfection efficiency and toxicity. In order to obtain a cleavable complex, we chose to use a SPDP linker which upon reaction with cystein-containing PNA will form disulfide linkages (Figure 1B−C), and we also decided to include a PEG containing SPDP linker for possible reduction of cellular toxicity. In efforts to quantify PEI modification, we found that authentic quantification using the standard TNBS assay (*34*) was not accurate, since amine derivatives of TNBS differ significantly in reaction kinetics and extinction coefficients depend on the compounds to react with TNBS (*35*). Furthermore, trinitrophenylation of PEI caused precipitation when the majority of primary amines were reacted. However, PEI precipitation was averted by supplementing the TNBS reaction (in borate buffer at pH 9.3) with 3% Tween 20, which did not change the absorbance of the reaction products with the simple PEI model compounds EDA and DETA (*36*), as compared to borate buffer alone (not shown). Under these conditions, absorbance at 420 nm correlated linearly with primary amine content of PEI, EDA, and DETA (Figure 2A), and eq 1 was derived ($R^2 = 0.997$, valid range = [5−110 μM]) from the standard curve. The absorbance at 420 nm was used because the absorption spectra of trinitrophenylated (TNP)-PEI, -DETA, and -EDA differed significantly at 340 nm and above 430 nm with TNP-PEI being a hybrid spectrum of TNP-DETA, TNP-EDA, and TNP-$NHCH_2CH_2NR_2$ (R ≠ H), but coalesced at 420 nm (Figure 2B).

$$[NH_2](\mu M) = \frac{(\text{Abs } 420\text{ nm}) - 85.5 \times 10^{-3}}{6.92 \times 10^{-3}} \quad (1)$$

The primary amine content of PEI25 was estimated to be ~175/polymer by comparison with TNP-DETA and TNP-EDA, which is in good agreement with the results of Harpe et al. (*14*).

Three preparations of sulfhydryl reactive 3-(2-pyridyldithio)-propionate (PDP) PEI differing in the degree of modification (14−68 per PEI) were made with both primary amine reactive SPDP and SPDP-PEG_8 linkers (Figure 1B). The conjugates were purified on Sephadex G-25SF preconditioned with PEI to block unspecific adsorption. Preconditioning the columns with PEI was critical in reducing nonspecific adsorption and significant sample loss, which was apparent when using a fluorophore-label PEI25 (not shown). We therefore assumed adsorption kinetics and capacity were analogous to those of cellulose fibers (*37*) and handled the beads accordingly, thereby reducing column loss to a minimum (as judged by control experiments using fluorescein-labeled PEI25 (results not shown).

The collected fractions were submitted to dialysis. The conjugates were characterized spectrophotometrically using the 3% Tween 20, borate buffered TNBS assay, and by measuring pyridine-2-thione (P2T) released by DTT treatment of the samples. The primary amine content ([NH_2]) was estimated from eq 1 and PEI concentration and the degree of modification

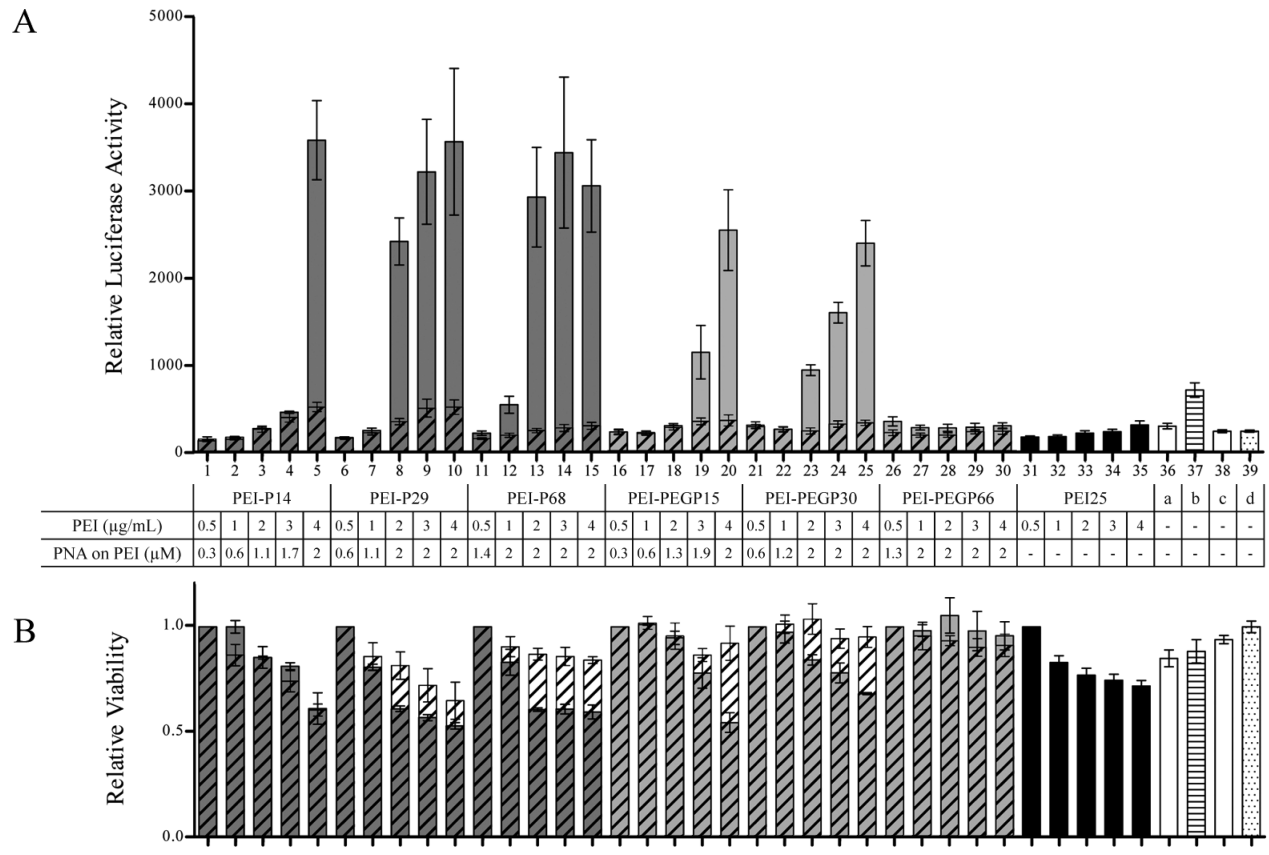

Figure 3. Effects of PEI modification. Transfections were carried out with a constant PNA concentration of 2 μM and varying concentration of functionalized PEI from 0.5 to 4 μg/mL. DTT controls were prepared for each sample (hatched overlay) in which PNA was cleaved from the PEI before transfection. Controls are 4 μM Arg_8-PNA (a), 4 μM Arg_8-PNA with chloroquine (75 μM) (b), 4 μM Cys-PNA (c), and 20 mM HEPES (1:3, v/v) in OPTI-MEM (d). Relative luciferase activity is corrected for ATP content. Error = SEM, $n = 3$.

({PDP/PEI}) from eqs 2 ([P2T] (μM) = (Δ343 nm)/(8.08 × 10^{-3})) and 3, respectively (Table 1).

$$[\text{PEI}] = \frac{([\text{NH}_2] + [\text{P2T}])}{175} \quad (2)$$

$$\{\text{PDP/PEI}\} = \frac{[\text{P2T}]}{[\text{PEI}]} \quad (3)$$

The modification degree and standard deviation (Table 1) were determined by unpaired comparison of triplicate TNBS and P2T release measurements, and the amine coupling efficiency (assuming 100% P2T cleavage) averaged 62%.

The PDP activated PEI was reacted with Cys-PNA (Figure 1C) in the wells in HEPES buffer and was used without further purification. Thus, samples were conveniently prepared in a 96-well format. We evaluated the transfection capacities of PEI-P/PEGP conjugates in the well-established HeLa pLuc705 splice correction assay (*38*) using a PNA targeted to the aberrant splice site (plucPNA; Ac-Cys-CCTCTTACCTCAGTTACA-NH_2), and the luciferase activity was corrected for cell viability using ATP content (rather than standard BCA assay), as this is more conveniently done in a 96-well format.

The conjugates were initially evaluated by keeping the total PNA concentration constant at 2 μM and varying the PEI concentration in the conjugation (Figure 3A). All samples were prepared with a corresponding DTT control, which was treated with 1 mM DTT prior to transfection to measure background transfection of nonconjugated PNA and PEI (Figure 3, hatched overlays). The results showed a very significant increase (up to <20-fold above background) in luciferase activity induced by the PNA upon conjugation to PEI, and this effect is virtually abolished after treatment with DTT, demonstrating the necessity of the covalent disulfide linkage for activity. The activity of the PNA-PEI conjugates is somewhat dependent on the degree of PEI modification (Figure 3A, PEI-P14, columns 1−5, vs PEI-P29 and -P68, columns 5−10 and 11−15, respectively), and the simple SPDP conjugates appear more active than the corresponding SPDP-PEG_8 conjugates (Figure 3A, PEI-P68 vs PEI-PEGP66, columns 1−15 and 16−30, respectively). In particular, the SPDP-PEG_8 conjugates are virtually inactive at the higher modification degree (Figure 3A, PEI-PEGP66, columns 26−30), although PEG-modification alleviates cellular toxicity to some extent. One possible explanation for this reduced efficiency of PEG-modified PEI is shielding of the PEI amines. Furthermore, all PEI preparations showed a dose-dependent cell toxicity (Figure 3B), and it is interesting that toxicity seems reduced upon treatment with DTT, indicating that the PNA conjugation increases the general cellular toxicity of PEI. From these results with constant PNA concentration for the conjugation (Figure 3), we found that the optimal PDP:PNA ratio was 1.2:1, which was therefore used in the dose−response experiments (Figure 4A−B).

The antisense activity of the apparently most potent and less toxic conjugates, PEI-P29, PEI-PEGP15, and PEI-PEGP30 were studied in more detail. A dose−response dependence on PNA concentration showed a close to linear relationship of all three conjugates, with the PEI-PEGP15 conjugate being twice as active as the two others most likely reflecting the reduced PEI loading and thus double PEI concentration (Figure 4A). Thus, the PEI-PEGP15 conjugate was chosen for a direct comparison with a reference CPP conjugated PNA, Arg_8-PNA (*6*−*8*) (Figure 4B). These results show that the PEI-PEGP15-PNA conjugate is about 10-fold more active than the Arg_8-PNA and up to 3−4-fold more active, even when the effect of Arg_8-PNA is enhanced with the lysosomolytic agent chloroquine (CQ) (*6*). Furthermore,

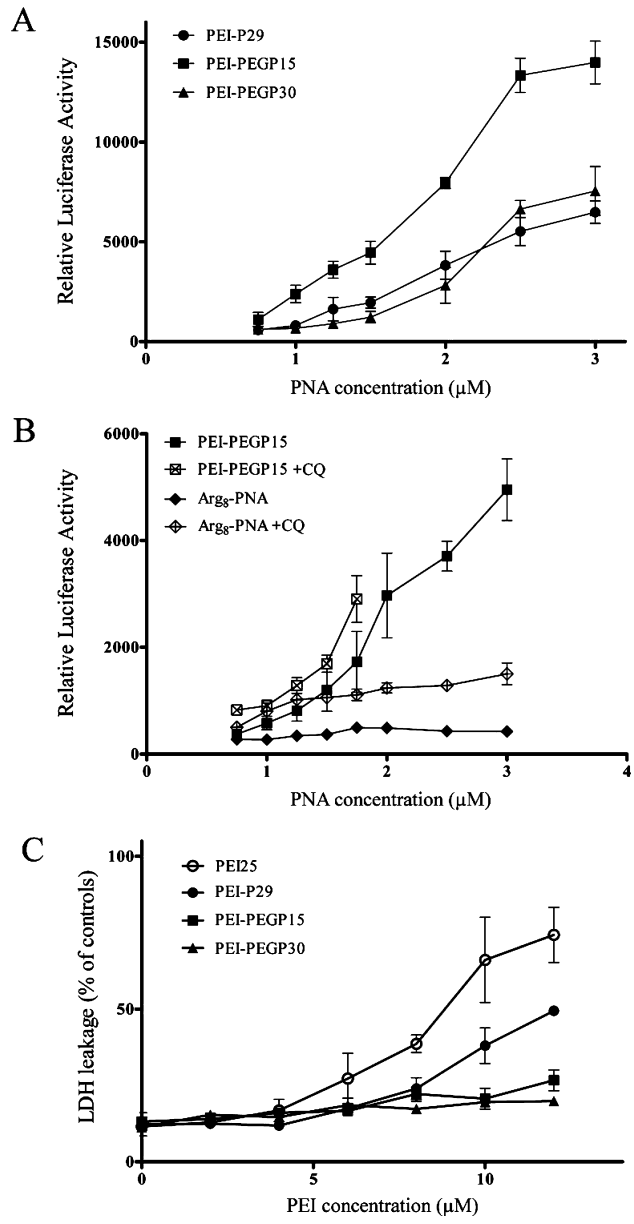

Figure 4. Dose response and acute toxicity. (A) Antisense activity of PEI-P29-, PEI-PEGP15-, and PEI-PEGP30-PNA conjugates. (B) Antisense activity of PEI-PEGP15-PNA conjugate and Arg_8-PNA in the absence or presence of chloroquine (CQ). CQ and PEI-PEGP15-PNA corresponding to PNA concentrations above 1.75 μM were highly toxic and are omitted. (C) Acute toxicity at 2 h as measured by LDH leakage. For (A) and (B), the PEI-P/PEGP concentration was determined by a PDP:PNA ratio of 1.2: 1. Relative luciferase activity is corrected for ATP content. Error = SEM, $n = 3$.

and most interestingly, the addition of chloroquine has only minimal activating effect on the PEI-PEGP15-PNA conjugate, but very significantly increases the toxicity of this (>50% for 2 μM PNA; Figure 4B).

Finally, whereas cell viability after 24 h (Figure 3B) as measured by the ATP assay did not differ much between PEI and PEI-PEG conjugates, the PEI-PEG conjugates showed significantly less acute toxicity (2 h) as measured by LDH leakage (Figure 4C). The reduced acute toxicity of SPDP-PEG_8 modified PEI25 together with the decrease in toxicity of DTT cleaved conjugates after 24 h of transfection, especially for PEI-PEGP30 and 66 (Figure 3B, columns 21−30), could indicate that the $[PEG]_8$ linker leads to reduced initial membrane damage (and possibly lower transfection efficacy when cleaved), while the delayed cytoplasmic toxicity could be caused by pertained mitochondrial damage (*19*). This is

further supported by the negligible difference between high and low modification degree with SPDP.

CONCLUSIONS

The present results clearly show that properly optimized, in terms of conjugation degree, PEI is an efficient cellular delivery agent for (antisense) PNA not requiring additional lysosomolytic activity. We also note that the experimental setup allows for high-throughput screening optimization and discovery of novel active PEI-conjugates using a variety of other bioactive ligands, such as morpholino oligomers (*39*) and minor groove binding (hairpin) polyamides (*40*). Furthermore, the system also conveniently allows for studies and optimization of heterogeneous conjugates using gene targeting agents (e.g., PNA) in combination with auxiliary agents, which have previously proven successful in improving transfection efficiency of PEI:DNA polyplexes, such as specific receptor targeting peptides (e.g., FGF) (*41, 42*), cell penetrating peptides (CPPs) (*43*−*45*), and lipids (e.g., cholesterol) (*46*), in order to optimize and tailor-make the conjugates, and exploiting the possible synergy of the agents. The ratio between the various ligands in the PEI conjugate will (to a first degree) be controlled by their ratio (as thiol derivatives) in the conjugation reaction mixture. Finally, the employment of orthogonal conjugation methods (e.g., click chemistry) would allow discrimination of ligands attached by cleavable or noncleavable linkers.

ACKNOWLEDGMENT

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