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# Cell number and transfection volume dependent peptide nucleic acid antisense activity by cationic delivery methods

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Efficient intracellular delivery is essential for high activity of nucleic acids based therapeutics, including antisense agents. Several strategies have been developed and practically all rely on auxiliary transfection reagents such as cationic lipids, cationic polymers and cell penetrating peptides as complexing agents and carriers of the nucleic acids. However, uptake mechanisms remain rather poorly understood, and protocols always require optimization of transfection parameters. Considering that cationic transfection complexes bind to and thus may up-concentrate on the cell surface, we have now quantitatively compared the cellular activity (in the pLuc705 HeLa cell splice correction system) of PNA antisense oligomers using lipoplex delivery of cholesterol- and bisphosphonate-PNA conjugates, polyplex delivery via a PNA-polyethyleneimine conjugate and CPP delivery via a PNA-octaarginine conjugate upon varying the cell culture transfection volume (and cell density) at fixed PNA concentration. The results show that for all delivery modalities the cellular antisense activity increases (less than proportionally) with increasing volume (in some cases accompanied with increased toxicity), and that this effect is more pronounced at higher cell densities. These results emphasize that transfection efficacy using cationic carriers is critically dependent on parameters such as transfection volume and cell density, and that these must be taken into account when comparing different delivery regimes.



## Introduction

Efficient intracellular delivery is essential for high activity of nucleic acid based therapeutics including antisense agents. In contrast to most small molecule drugs which are taken up by cells via (passive) diffusion through the lipid cell membrane, oligonucleotides and other antisense oligomers, such as peptide nucleic acids (PNAs) and phosphorodiaminidate morpholino oligomers (PMOs), require a carrier for cellular uptake due to their large size and hydrophilic character (in the case of oligonucleotides even high negative charge). Effective delivery vehicles are typically polycationic, in the form of cationic lipids, polyamines or (cationic) cell penetrating peptides. These vehicles form complexes with the negatively charged oligonucleotides, but the resulting lipoplexes and polyplexes also require an overall positive zeta potential for efficacy. The positive charge enables the carrier to interact with the cell membrane and internalize into the cytosol, generally via endocytotic pathways.[1]

Following cellular uptake, lipoplexes destabilize the endosomal membrane, resulting in a flip-flop reorganization of phospholipids. These phospholipids then diffuse into the lipoplex and interact with the cationic lipids, causing the DNA to dissociate and diffuse into the cytosol.[1] Lipoplexes have successfully been employed for cellular delivery of DNA and nucleic acid analogs by complexation via electrostatic interaction. However, charge neutral antisense oligomers like peptide nucleic acids (aegPNAs) or PMOs do not interact with the cationic lipid themselves, and several modifications have been developed in order to increase the affinity of the PNA for the cationic liposome carrier. Lipophilic or negatively charged moieties have been conjugated to PNA to facilitate hydrophobic or ionic interactions, respectively, with the cationic lipids, and PNA/DNA heteroduplexes have been prepared with partially overlapping single-stranded carrier DNA which then promotes complexation.[2] Examples of hydrophobic/anionic PNA conjugates which have shown potent transfection efficiency are the introduction of negative charges through coupling with bis-phosphonate[3] and lipid conjugation to fatty acids, heteroaromatic compounds, cholic acid or cholesterol moieties.[4-6]

An alternative to cationic lipids is the use of cationic polymers that can be complexed (or chemically conjugated) to the nucleic acid, forming cationic polyplexes (or nanoparticles). The most extensively used cationic polymer for transfection is poly(ethylenimine) (PEI).[7] The high amine content and buffering capacity of PEI is hypothesized to be the main driving force of its efficiency, resulting in an endosomal import of protons, high

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osmotic pressure and subsequent rupture of the endosomes without need of additional endosomolytic activity. PEI is commercially available in a large molecular weight range and various degrees of branching making it highly versatile in preparations. Furthermore, it is readily modified by various chemical strategies to alter its physicochemical character or to confer transfection specificity by attaching ligands for cell targeting.[1] Also, PEI has been shown to increase transfection efficacy when conjugated to a PNA oligomer via an intracellularly cleavable linker.[8]

Cell penetrating peptides (CPPs), a group of cationic peptides typically containing multiple arginines, are effectively taken up by eukaryotic cells and can be used to deliver conjugated molecular cargos into the cell.[9] The initial reports suggested that positively charged CPPs interact with negatively charged components of the membrane, like heparin sulfate, as well as the phospholipid bilayer, and were internalized by membrane destabilization or pore formation. A large number of recent studies have, however, demonstrated that cellular uptake of CPPs mainly takes place by endocytotic pathways.[10] Conjugation to such peptides is also a useful technique for the delivery of peptide nucleic acids, presenting several advantages like availability through continuous solid phase-synthesis and avoidance of lipid transfection reagents. However, endosomal escape is generally limited and requires the use of endosomal disrupting agents like chloroquine, calcium ions, lipophilic photosensitizers or sucrose.[10-12] Furthermore, chemical modifications of CPPs, such as conjugation to a lipid domain (CatLip conjugate), have been found to significantly improve their delivery efficacy.[13-15]

Cationic lipoplexes and polyplexes associate with the cell surface, and such up-concentration may result in enhanced cellular uptake.[15,16] This phenomenon has also been observed for cell penetrating peptides, for which cellular uptake exhibited volume-dependent behavior.[17] In order to directly compare this effect on different delivery modalities we have taken advantage of the antisense system based on PNA targeting to the cryptic splice site within the mutated luciferase gene in HeLa pLuc705 cells.[18] This system permits the use of different delivery vehicles with PNA oligomers optimized to the delivery agent but of identical sequence. Thus we have employed lipoplex delivery of cholesterol-PNA and bisphosphonate-PNA conjugates, polyplex delivery via a PNA-PEI conjugate and CPP delivery via a PNA-octaarginine conjugate, and quantitatively compared the antisense effects upon varying the cell culture transfection volume (and cell density) at fixed PNA concentration.

The results show that for all delivery modalities the cellular antisense activity increases (less than proportionally) with increasing volume and that this effect is more pronounced at higher cell densities. These results emphasize that transfection efficacy using cationic carriers is critically dependent on parameters such as transfection volume and cell density, and that these must be taken into account when comparing different delivery regimes.

## Results

A large number of methods are available for nucleic acid cellular transfection and typically require careful optimization for use. However, despite the fact that cationic transfection reagents cause up-concentration of the (nucleic acid) cargo on the cell membrane, the effect of the volume of the transfection medium is rarely considered in experimental protocols. Thus in a comparative study across different cationic delivery modalities we set out to study this effect using different volumes of PNA-lipoplex, -polyplex or PNA-CPP preparations exploiting different PNA modifications (**Table 1**) designed for use with these delivery modalities.

In order to define initial conditions, PNA doses, transfection reagents and incubation time were individually optimized for each PNA modification according to transfection efficacy and toxicity data. Conditions showing the highest antisense activity above 50% cell viability were selected for further volume-effect assays (data not shown). Subsequently, transfection solutions were prepared specifically for each PNA conjugation. PNAs were either complexed with a cationic lipid reagent (Lipofectamine 2000), chemically conjugated to a cationic polymer (PEI), or for CPP-PNAs, mixed with CQ as a singular preparation at their optimal concentrations and transfected to HeLa pLuc705 cells for 24 h. All tested PNA modifications contained a PNA oligomer of identical sequence targeting the cryptic splice site within the luciferase gene in HeLa pLuc705 cells and antisense activity (and thus be inference cellular uptake) was measured as induced luciferase activity.[18]

In view of the previous evidence of the up-concentration of cell-penetrating peptides on the cell surface,[17] our first choice for studying the volume effect of transfection solutions was the CPP-PNA conjugate. First we examined the effect of the CPP-PNA modification on HeLa pLuc705 cells at 4 μM PNA and $8 \times 10^3$ cells/well (in order to have 50% confluency at the transfection time, which is the typical cell confluency for ordinary



**Table 1.** List of PNAs

| PNA No | Name | Sequence* |
|---|---|---|
| 2977 | Chol-hs-PNA | Cholesteryl hemisuccinate-CCT CTT ACC TCA GTT ACA-$NH_2$ |
| 3325 | bP6-PNA | H-[Lys-(Bis-phosphonate)]$_6$-CCT CTT ACC TCA GTT ACA-$NH_2$ |
| 2787 | (D-Arg)$_8$-PNA | H-(D-Arg)$_8$-Gly-CCT CTT ACC TCA GTT ACA-$NH_2$ |
| 3140 | PEI-PNA** | $PEI_{25K}$-($PEG_8$-SS-Ac-Cys-CCT CTT ACC TCA GTT ACA-$NH_2$)$_{15}$ |

*The sequences of the PNAs are written from N-terminal to C-terminal end. **Primary amines were reacted with SPDP-$PEG_8$ reagent containing an N-hydroxysuccinimide (NHS) ester and a sulfhydryl-reactive pyridyl disulfide group which could further be reacted with a cysteine-PNA. $PEI_{25}$k was on average modified with 15 SPDP-$PEG_8$ per polymer.

transfection). Under these conditions hardly any volume effect was observed (**Fig. 1**). This lack of effect could be due to saturation of the cells at this PNA concentration, and we therefore decided to increase the cell number range in steps from 4 to 32 × $10^3$ cells/well in order to obtain lower PNA-to-cell ratios. By using 24 or 32 × $10^3$ cells/well we observed around 2-fold efficacy increase at six relative volumes (**Fig. 1**), but cell viability was also reduced comparably (**Fig. S1**). These results do confirm that transfection efficacy depends on PNA-to-cell ratio, in accordance with previous conclusions,[16,17] and also indicate that saturation may be possible at lower cell numbers.

Next we analyzed an anionic PNA conjugate which can form a complex with cationic lipids in analogy to negatively charged nucleic acids.[3] A bis-phosphonate-PNA conjugate was transfected at different concentrations (0–2 nM) using varying volumes of the transfection solution/well in a 96 well plate seeded at 24 × $10^3$ cells/well. Results showed that antisense activity increased in a PNA dose-dependent manner at all transfection volumes, and in parallel, increasing volume also showed a higher antisense activity up to four relative volumes where a saturation was reached. We also observe from these experiments that the volume-effect is more pronounced at higher PNA concentrations. Whereas antisense activity at 1 and 2 nM PNA doses showed an approximately 2-fold increase at 4 relative volumes, at 0.5 and 0.25 nM PNA doses it was only about 1.75- and 1.5-fold, respectively (**Fig. 2**). However, the difference in PNA-Lipofectamine ratio, resulting in decreasing zeta potential as the PNA concentration increases at fixed Lipofectamine concentration, may also play a role, although an opposite effect could be expected as lowering of the zeta potential would decrease transfection efficacy. Cell viability exhibited a slight decrease (< 30%) with increasing transfection volume, but showed no dependence on PNA concentration (**Fig. S2**).

As an example of a PNA polyplex for which the zeta potential is only marginally affected by the PNA we chose a cholesteryl-PNA conjugate (PNA 2977, **Table 1**).[6] For this PNA modification we also observed a clear volume-dependent antisense activity at both 30 and 100 nM concentration (**Fig. 3**). Furthermore, the activity increased nearly linearly with the LFA2000 concentrations (2, 4 and 6 μl/ml), whereas only minor effects were observed on cell viability even for the highest PNA dose (**Fig. S3**). However, while at 30 nM PNA concentration the transfection efficacy was increasing for all relative volumes, this was limited to three relative volumes at 100 nM (**Fig. 3**).

In order to test the generality of the effect, two of the PNAs, CPP-PNA and cholesteryl-PNA, were transfected at the same conditions in a 24-well plate (instead of 96-well plate) with

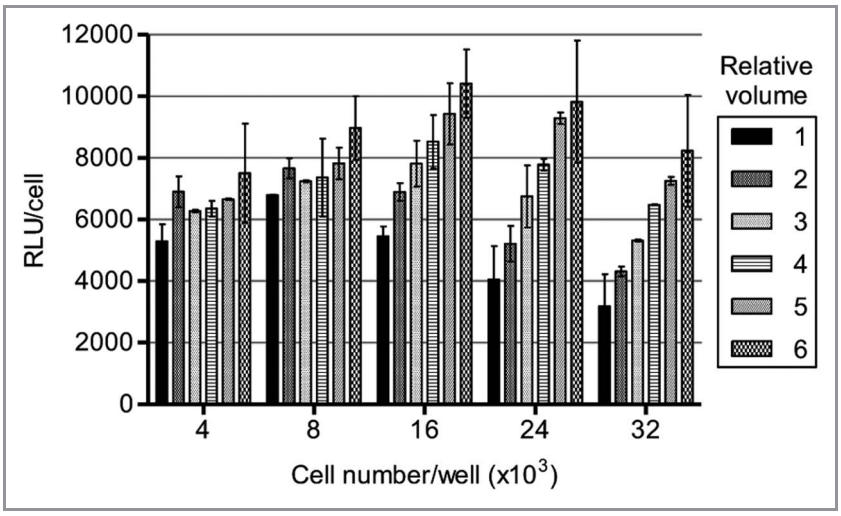


**Figure 1.** Relative cellular luciferase antisense activity in HeLa pLuc 705 cells of PNA conjugated to octa-arginine [$(D\text{-}Arg)_8$-PNA]. Different cell numbers were plated in a 96-well plate the day before transfection. Cells were treated with different volumes (50 μl/well for a relative volume 1) of the transfection solution for 24 h: 4 μM PNA and 120 μM CQ. Cell samples were then subjected to luciferase analysis and cellular viability test. All tests were performed in duplicate and the results are given as average values ± standard deviations (SD). Luciferase activity was analyzed using Bright-Glo reagent (Promega), normalized to cell viability (**Figs. S1–5**) and given as relative light units (RLU/cell).

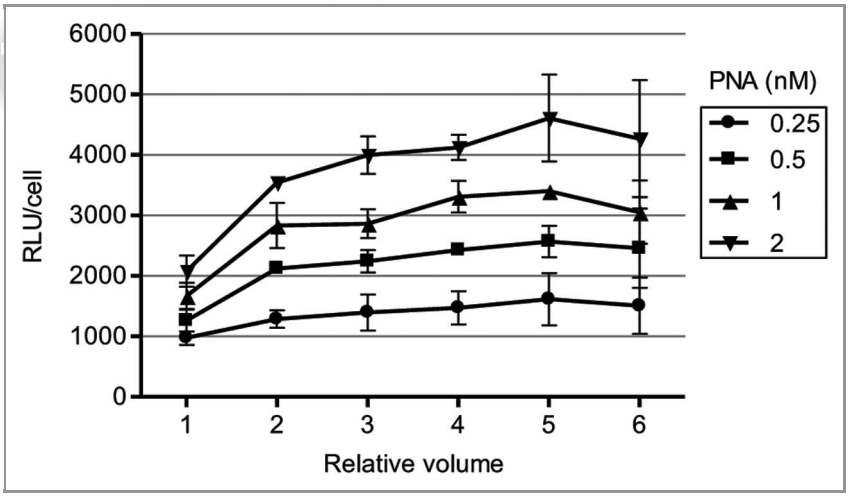


**Figure 2.** Relative cellular luciferase antisense activity of phosphonate-conjugated PNA (bP6-PNA). HeLa pLuc705 cells were trypsinized and seeded (2.4 × $10^4$ cells/well) in a 96 well plate the day before transfection. Cells were treated with different volumes (50 μl/well for a relative volume 1) of the transfection solutions for 24 h: 0.25 to 2nM PNA complexed with 6 μl/ml LFA2000. Cell samples were then subjected to luciferase analysis and cellular viability test. All tests were performed in triplicate and the results are given as average values ± standard deviations (SD). Luciferase activity was analyzed using Bright-Glo reagent (Promega), normalized to cell viability (**Figs. S1–5**) and given as relative light units (RLU/cell).

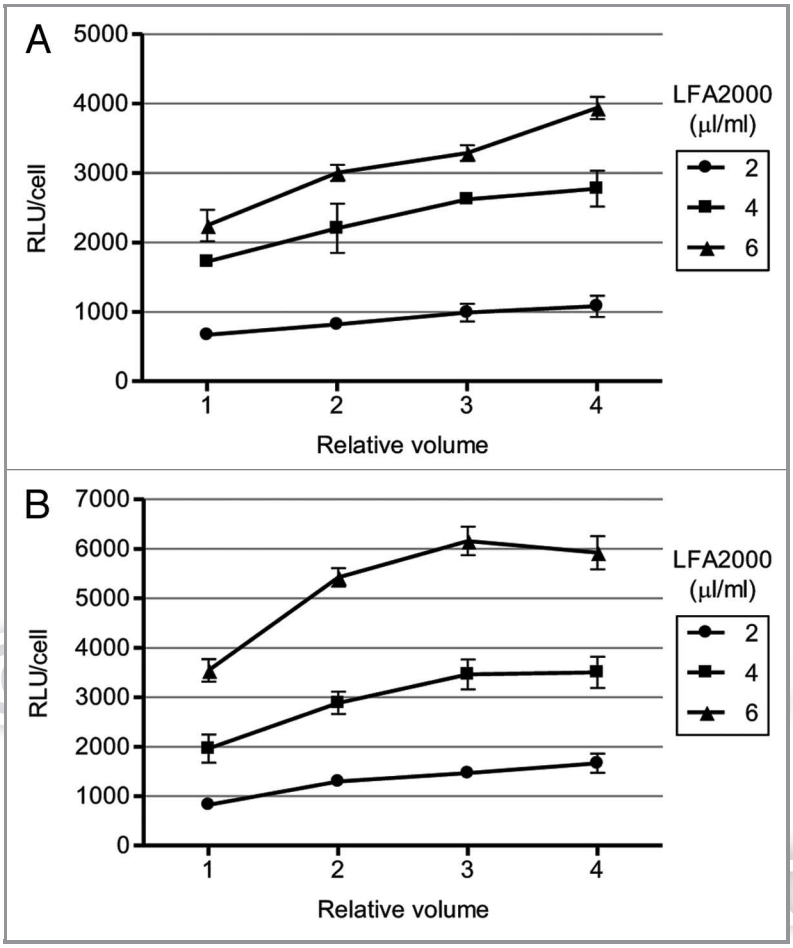


**Figure 3.** Relative cellular luciferase antisense activity of PNA conjugated to cholesteryl hemisuccinate (Chol-hs-PNA). HeLa pLuc705 cells were trypsinized and seeded ($8 \times 10^4$ cells/well) in a 96 well plate the day before transfection. Cells were treated with different volumes (50 µl/well for a relative volume 1) of the transfection solutions for 24 h: (A) 30 nM and (B) 100 nM PNA complexed with 2, 4 or 6 µl/ml LFA2000. Cell samples were then subjected to luciferase analysis and cellular viability test. All tests were performed in triplicate and the results are given as average values ± standard deviations (SD). Luciferase activity was analyzed using Bright-Glo reagent (Promega), normalized to cell viability (**Figs. S1–5**) and given as relative light units (RLU/cell).

equivalent cell confluency. Both conjugates showed an increase of the antisense activity with increasing transfection volume (**Fig. S5**), fully consistent with the results obtained in the 96 well plates.

Finally, we studied the volume-effect for a PNA-polyplex using a disulfide coupled PNA-PEI (polyethyleneimine) conjugate.[8] A 4-fold antisense activity increase at 5 relative volumes was seen for both of the analyzed doses (1 and 2 µM), but in contrast to the other PNA conjugates, the transfection volume-effect for the PEI-PNA is not dependent on the PNA dose (at 1 and 2 µM), and showed a close to proportional dependence on the volume up to 4 relative volumes (**Fig. 4**), whereas only minor effect of the cell number was observed. Finally, the PNA-polyplexes exhibited a pronounced volume-dependent cellular toxicity at the lower cell densities (4 and $8 \times 10^3$ cells/well) and the higher concentration (2 µM) (**Fig. S4**).

## Discussion

The present results unequivocally demonstrate that antisense activity increases by increasing the volume of the transfection solution for all four PNA complexes and conjugates, supporting that cationic PNA-lipolexes, PNA-PEI polyplexes as well as PNA-CPP conjugates have high affinity for the cell surface and that such cellular binding is important, or even necessary, for cellular uptake. This is fully consistent with previous reports, demonstrating that cell uptake of DNA can be enhanced by an increase of nucleic acid concentration on the target cell surface.[16]

Although a slight volume-dependent decrease in cell viability was observed under some conditions, the results show that lower PNA doses could be used at higher volumes to obtain higher transfection efficiency without concomitant increase in cellular toxicity.

These results emphasize that transfection efficacy using cationic carriers is critically dependent on parameters such as transfection volume and cell density, and that these can be optimized and must be taken into account when comparing different delivery regimes.

We believe that these conclusions are also valid to any nucleic acid transfection (DNA, RNA and analogs) that is performed by cationic transfection reagents (cationic lipids, cationic polymers or CPP conjugation).



## Materials and Methods

**Cell line and cultura media.** HeLa pLuc705 cells[18] were purchased from Gene Tools (Philomath). Cells were grown in RPMI1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma), 1% glutamax (Gibco, Invitrogen), and 100 µg/ml streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% $CO_2$.

**PNA synthesis.** Nomenclatures and sequences of the PNAs used in this study are listed in **Table 1**. PNA synthesis was performed by the standard Boc strategy as reported previously.[19] The resulting PNA conjugates were purified by HPLC and characterized by MALDI-TOF mass spectrometry. The PNAs were lyophilized and stored at 4°C until use.

*CPP-PNA.* Peptides were linked to the PNA at the N-terminal through glycine via continuous solid phase synthesis.[13]

*Bis-phosphonate-PNA.* Lysine bis-phosphonate monomers were covalently linked to PNA at the N-terminal on the solid support via continuous synthesis using Fmoc-chemistry.[3]

*Cholesterol-hemisuccinate-PNA.* Cholesterol-hemisuccinate was covalently linked to the PNA N-terminal amino group via solution conjugation.[6]

*PEI-PNA.* PEI-PEGP15 was prepared from 25 kDa (Mw) branched polyethylenimine (Sigma) and a heterobifunctional linker (amine- and thiol-reactive) with a discrete 8-mer PEG spacer, SPDP-$PEG_8$ (Quanta Biodesign) as previously described.[8]

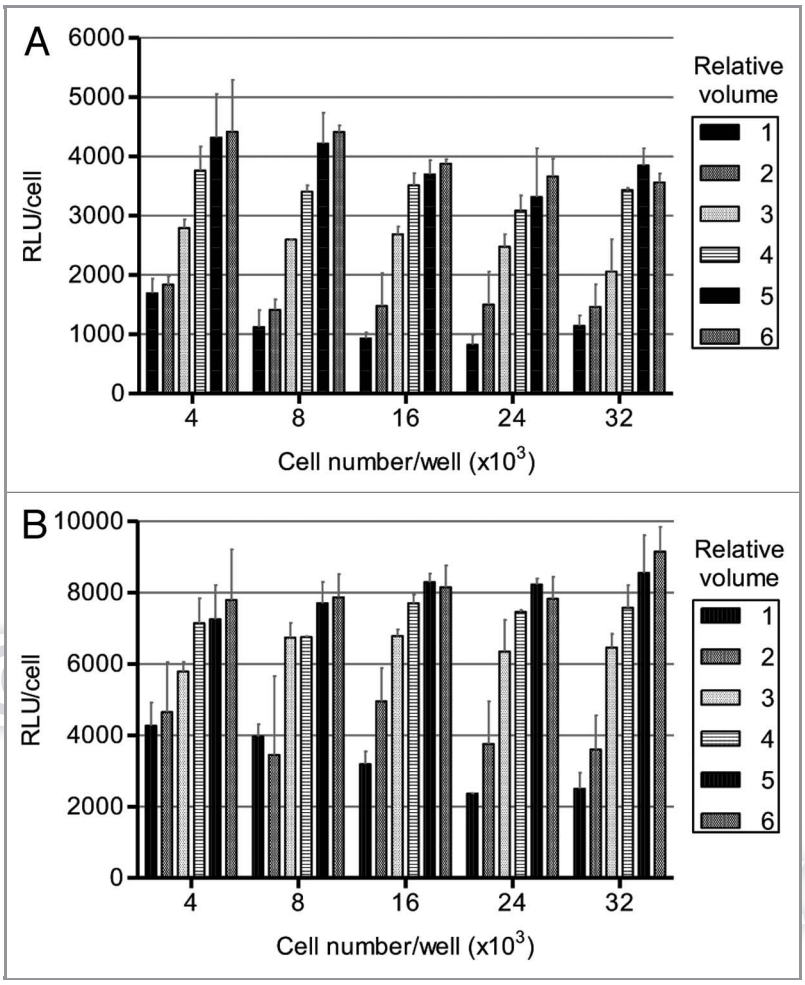


**Figure 4.** Relative cellular luciferase antisense activity of PNA conjugated to PEI [$PEI_{25K}$-($PEG_8$-SS-PNA)$_{15}$]. HeLa pLuc705 cells were trypsinized and seeded in a 96 well plate at different cell numbers the day before transfection. Cells were treated with different volumes (50 µl/well for a relative volume 1) of the transfection solutions for 24 h: (A) 1 µM or (B) 2 µM PNA (PDP:PNA; 1:1). Cell samples were then subjected to luciferase analysis and cellular viability test. All tests were performed in triplicate and the results are given as average values ± standard error of the mean (SEM). Luciferase activity was analyzed using Bright-Glo reagent (Promega), normalized to cell viability (**Figs. S1–5**) and given as relative light units (RLU/cell).

Briefly, 5 mg PEI (~0.2 µmol calc. from Mw or ~35 µmol R-$NH_2$) in 1.8 mL phosphate buffer, pH 7.4, was mixed with 5 µmol SPDP-$PEG_8$ (theo. twenty-five $PEG_8$-PDP per PEI) in 200 µl DMSO and incubated at RT for 8 h. The product was dialyzed (Spectra/Por 2, MWCO: 12–14 kDa, Spectrum Laboratories) and characterized by spectrophotometric quantification of pyridine-2-thione released by DTT cleavage of the linker and quantification of primary amines in PEI with a TNBS assay. This was subsequently used to determine a modification degree of averagely 15 $PEG_8$-PDP per PEI (PEI-PEGP15). The $PEG_8$-PDP modified PEI was kept in freeze stock until it was conjugated to Cys-PNA just before transfection (see 'PNA transfection').

PNA stock solutions at 200 µM in water were kept at 4°C until use (Cys-PNA was stored at -20°C and kept at 4°C until immediately before coupling to PEI).

**PNA transfection.** Exponentially growing cells were cultured with antibiotic-free growth medium in 96- or 24-well plates the day before transfection at different cell numbers.

*Cholesterol and phosphonate PNAs.* Lipofectamine2000 (LFA2000, Invitrogen) was used for the transfection of the PNAs with Cholesterol (PNA2977) and phosphonate (PNA3325) modifications. LFA2000 was diluted with OPTI-MEM (Gibco) and mixed with an equal volume of the PNA in $H_2O$ solution to the desired concentrations; being ¼ of the final transfection volume. This solution was incubated for 10 min at room temperature and then mixed with RPMI1640 medium without antibiotics (¾ of the final transfection volume) to the desired final volume. Growth medium was removed from cells and replaced by the transfection solution. Cells were incubated for 24 h at 37°C and subjected to further analysis.

*Cell-penetrating peptide-PNA.* CPP-PNA (PNA2787) was diluted in $H_2O$ at the desired concentrations, to a ¼ of the final transfection volume. Chloroquine (CQ, Sigma) was diluted in OPTI-MEM to a ¾ of the final volume, and mixed with the PNA solution. Growth medium was removed from cells and replaced by the transfection solution for 4 h at 37°C. After PNA treatment, cells were supplemented with 10% FBS and incubated further for 20 h without removing the transfection solution.

*PEI-PNA.* Cys-PNA (PNA3140) was left to react with PEI-PEGP15 in HEPES (20 mM, pH 7.4) for one hour ($PEG_8$-PDP:PNA ratio = 1:1) to create $PEI_{25K}$-($PEG_8$-SS-PNA)$_{15}$. Then ¼ volume of $PEI_{25K}$-($PEG_8$-SS-PNA)$_{15}$ was mixed with ¾ of OPTI-MEM. Growth medium was removed from cells and replaced by the transfection solution for 4 h at 37°C. After PNA treatment, cells were supplemented with 10% FBS and incubated further for 20 h without removing the transfection solution.

**Luciferase assay.** Twenty-four hours after treatment, cells were subjected to luciferase activity analysis by using the Bright-Glo Luciferase assay system (Promega). Measurements were performed according to the manufacturer's instructions. Luminescence readings obtained with the Bright-Glo Luciferase assay system are presented as relative light units (RLU). Cells were lysed with 100 µl/well of Passive Lysis Buffer (Promega) for 10 min and used for both luciferase and viability assays. Luminescence readings were normalized by cell viability.

**Luciferase activity normalization.** For cells transfected on 96 well plates luciferase activity was normalized by cell viability and shown as RLU/cell; Ten microliters of cell lysate were diluted with 40 µl $H_2O$ and analyzed for ATP content by using CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's directions. The values were presented as relative cellular viability (the value from non-PNA treated cells was set as 100%).

For cells transfected on 24-well plates, luciferase activity was normalized by protein concentrations, which were determined by the BCA protein assay (Pierce) according to the manufacturer's instruction.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


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### Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/artificialdna/article/19906

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