Efficiency of Cellular Delivery of Antisense Peptide Nucleic Acid by Electroporation Depends on Charge and Electroporation Geometry

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Electroporation is potentially a very powerful technique for both *in vitro* cellular and *in vivo* drug delivery, particularly relating to oligonucleotides and their analogs for genetic therapy. Using a sensitive and quantitative HeLa cell luciferase RNA interference mRNA splice correction assay with a functional luciferase readout, we demonstrate that parameters such as peptide nucleic acid (PNA) charge and the method of electroporation have dramatic influence on the efficiency of productive delivery. In a suspended cell electroporation system (cuvettes), a positively charged PNA (+8) was most efficiently transferred, whereas charge neutral PNA was more effective in a microtiter plate electrotransfer system for monolayer cells. Surprisingly, a negatively charged (−23) PNA did not show appreciable activity in either system. Findings from the functional assay were corroborated by pulse parameter variations, polymerase chain reaction, and confocal microscopy. In conclusion, we have found that the charge of PNA and electroporation system combination greatly influences the transfer efficiency, thereby illustrating the complexity of the electroporation mechanism.

Introduction

IT IS GENERALLY ACKNOWLEDGED that efficient *in vivo* delivery and sufficient bioavailability at the molecular intracellular targets of RNA interference compounds is still a major challenge in need of novel solutions (Corey, 2007). This is also very much the case concerning compounds based on peptide nucleic acid (PNA) (Nielsen et al., 1991), a synthetic DNA mimic having a charge neutral pseudopeptide backbone, which in the original form consists of aminoethylglycine units replacing the anionic sugar phosphate backbone of nucleic acids (Koppelhus and Nielsen, 2003; Lundin et al., 2006). Apart from inherent charge neutrality, the structure of the PNA may give the molecule several advantages over oligonucleotide-type compounds. Most important is the very high biological (and chemical) stability as well as easy access to a wide range of chemical modifications in a medicinal chemistry context. However, despite these apparent advantages, biological applications of PNA molecules are limited by their inherently poor cellular uptake, being a relatively hydrophilic molecule that does not readily cross cell membranes (Wittung et al., 1995). Although several delivery methods have been described to enhance cellular uptake *in vitro*, including microinjection (Hanvey et al., 1992), electroporation (Karras et al., 2001), incorporation into liposomes (Hamilton et al.,

1999; Shiraishi et al., 2008), and conjugation to cell penetrating peptides (Koppelhus and Nielsen, 2003; Bendifallah et al., 2006; Mae and Langel, 2006; Lebleu et al., 2008) or receptor targeted ligands (Basu and Wickstrom, 1997; Zhang et al., 2001), and several studies have demonstrated *in vivo* effects in animal models of PNA conjugates (Sazani et al., 2002; Ivanova et al., 2008), a major challenge still exists in finding effective and easy *in vivo* delivery methods of PNA to be able to reach clinical trials and eventually clinical use.

Electroporation exploits short, intense electric pulses to create transient and reversible permeabilization of the cell membrane (Gehl, 2003), thereby promoting uptake of otherwise nonpermeating molecules. By applying an external field, an altered transmembrane potential in a cell is induced, and when the transmembrane potential net value (the sum of the applied and the resting potential difference) is larger than a threshold, transient permeation structures are generated in the membrane and electroporation is achieved (Gehl, 2003). Experimentally, optimization of pulses is essential for achieving high cell transfection combined with high cell viability, and optimal electroporation conditions depend on, for example, cell type and the type of molecules to be transfected (Gehl et al., 1998). Following electroporation, diffusion is responsible for the uptake of small molecules into the cell, and the higher the pulse amplitude applied, the greater will be the

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area over which diffusion can take place (Gabriel and Teissie, 1997; Neumann et al., 1998). In the case of larger, charged molecules such as DNA, electrophoretic forces play an important role in driving the polyanionic DNA toward the positive electrode and into the cell (Klenchin et al., 1991; Gabriel and Teissie, 1997; Zaharoff et al., 2002). Thus, the charge of a molecule very significantly will influence the efficiency and mechanism of electrotransfer.

The chemical properties of PNA, on one hand, allow charge optimization of the transfection by using identical sequences, but differently charged PNA conjugates, on the other hand, provide the basis of a controlled system to systematically study the effect of charge on the electrotransfer process under different conditions. We have exploited the quantitative and very sensitive pLuc705 luciferase HeLa cells assay (Kang et al., 1998) to study the delivery of 4 differently charged PNA oligomers targeted to the Luc705 splice junction. As in previous studies (Bendifallah et al., 2006; Shiraishi and Nielsen, 2006; Shiraishi et al., 2008), we used the mRNA splice correction and thus expression of functional luciferase as a measure of functional cellular uptake.

We chose to study previously well-characterized PNAs in the form of a charge neutral, unmodified PNA; a negatively charged (−23) PNA–phosphonate conjugate emulating an anionic oligonucleotide (Shiraishi et al., 2008); and 2 positively charged PNA–CPP (cell penetrating peptide) conjugates (Bendifallah et al., 2006) (with charge +8 for an Arg₇, and +5 for an Lys₄ conjugate). We also employed and optimized 2 different electroporation setup geometries, one developed for cells in suspension and another for monolayer cells.

Interestingly, we found dramatic differences in electrophoretic delivery efficiency between the differently charged PNAs, and most surprisingly, we found that the charge neutral PNA was most effectively delivered in the monolayer setup, whereas the cationic Arg₇–PNA conjugate was most efficiently delivered in the suspension setup. The results bear on the mechanisms of electrophoretic cellular delivery and also illustrate the possibilities of chemical (or formulation) optimization in terms of the active agent.

Materials and Methods

Cell culture

HeLa pLuc 705 cells are stably transfected with a recombinant plasmid (pLuc/705) carrying a luciferase gene interrupted by a β -globin intron 2 mutated at nucleotide 705, thereby introducing an aberrant splice site (Kang et al., 1998). Cells were grown in Dulbecco's modified Eagle's media (Lonza) with 15 mM Hepes and L-glutamine, supplemented with 10% fetal bovine serum, 10,000 U/mL penicillin, and 10,000 U/mL streptomycin at 37°C under 5% CO₂. Cells were harvested using trypsin/EDTA (Lonza).

Peptide nucleic acids

All the PNAs have the same base sequence targeting the aberrant splice site at nucleotide 705 in the β -globin intron 2. The PNAs were synthesized, purified, and characterized as previously reported (Christensen et al., 1995; Shiraishi and Nielsen, 2006; Shiraishi et al., 2008). The PNAs were dissolved in sterile water and the concentration was determined spectrophotometrically using the extinction coefficient 177,200 L/

(mol cm) at 260 nm. PNAs with different charges (at neutral pH) were included in the experiments, namely PNA 3325: H-(Lys(bisP-4))₆-CCT CTT ACC TCA GTT ACA-NH₂ (23 negative charges, molecular weight: 6911.96 g/mol); PNA 2392: H-(Arg)₇-eg1-CCT CTT ACC TCA GTT ACA-NH₂ (8 positive charges, molecular weight: 6003.89 g/mol); PNA 2870: Ac-eg1-CCT CTT ACC TCA GTT ACA-NH₂ (charge neutral, molecular weight: 4953 g/mol); PNA 3333: H-(D-Lys)₄-CCT CTT ACC TCA GTT ACA-NH₂ (5 positive charges, molecular weight: 5278.13 g/mol); PNA 2817: H-Flk-CCT CTT ACC TCA GTT ACA-NH₂ (charge neutral, fluorescein conjugate, molecular weight: 5294.06 g/mol); and PNA 3266: H-Flk-(D-Arg)₈-AAT CTC ACC TGA TAG T-NH₂ (8 positive charges, fluorescein conjugate, molecular weight: 6107.08 g/mol) [Flk is a fluoresceinated lysine (Lohse et al., 1997)].

Electrotransfer

Two different electrotransfer equipments were used (Fig. 7): a cuvette system (Cliniporator; IGEA) and Cellaxess CX3 (Celletricon). The 2 electroporation systems used differ in geometry and mode of operation.

With the Cellaxess CX3 system, it is possible to apply the electrical field directly in 96-well plates (no cuvettes needed) and 3 parallel wells can be electroporated simultaneously. In this system, the PNA is flushed in for 60 seconds and electroporation is simultaneously applied during the last 10 seconds of flushing. The electroporation device consists of 3 independent circular electrodes and the electrical field in this device has the highest field strength in the center and declines as $1/r$ toward the outer rim of the electrode (Olofsson et al., 2005). The transfection of cells in 96-well plates leads to a decrease of the area in which cells are actually exposed to electroporation. The area is highly cell-type dependent but is approximately 20–50% of the total area (Olofsson et al., 2005).

In the cuvette system, cells are electroporated in suspension in electroporation cuvettes that are designed with 2 aluminium plate electrodes covering 2 sides of the cuvette with a gap size of 4 mm. In this system, a rather homogenous field is obtained between the 2 electrodes (Gehl et al., 1999).

With the cuvette system, cells were harvested, centrifuged, and resuspended in phosphate-buffered saline (Hospital Pharmacy) to 2×10^5 cells/mL. Four-hundred microliters of the cell suspension was transferred to each electroporation cuvette (Thermo Fisher Scientific) and PNA was added to a final concentration of 1 μ M. Electroporation was performed with settings of 5 pulses of 100 V/cm for 5 ms and a frequency of 1 Hz or 6 pulses of 1000 V/cm for 0.1 ms and 1 Hz. After electroporation, the cuvettes were incubated at 37°C under 5% CO₂ for 30 minutes before handling. From each cuvette, 200 μ L of cell suspension was transferred to 24-well plates and grown for 24 hours, and luciferase activity was measured. With the Cellaxess CX3 equipment, cells were harvested and plated in 96-well plates with 10,000 cells per well and grown overnight. Electrotransfer of PNA at a concentration of 1 μ M was performed with settings of 10 pulses of 120–250 V for 1–50 ms and a frequency of 1 Hz. After electroporation, the cells were incubated for 24 hours at 37°C under 5% CO₂ and luciferase activity was measured.

Luciferase splice correction assay

Uptake of productive PNA in the cells was measured as induced luciferase activity in HeLa pLuc705 cells (which are

stably transfected with a recombinant luciferase gene) (Kang et al., 1998). The recombinant luciferase gene carries an alternative splice site leading to aberrant splicing, so that hardly any full-length, functional luciferase protein is expressed. Properly internalized PNA will block the alternative splice site and induce correct splicing and thereby luciferase expression. The measured luciferase activity is therefore an indirect measurement of functional PNA in the nucleus of the cells.

At 24 h after electroporation, luciferase activity in the cells was measured using Luciferase Reporter Gene Assay, high sensitivity (Roche Diagnostics), according to the manufacturer's instructions. After lysis of the cells, the solution was transferred to 96-well plates, and luciferase activity was measured. Luminescent readings were performed on an Infinite M200 luminometer (Tecan) and LUMIstar Optima microplate reader (BMG LABTECH), respectively. Luminescent readings are presented as relative light units, and cells exposed to electroporation and PNAs were compared with untreated controls.

Reverse transcription–polymerase chain reaction

RNA was extracted from cells using Passive Lysis Buffer and RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Four nanograms of RNA was used for each reverse transcription (RT)–polymerase chain reaction (PCR) reaction using OneStep RT-PCR kit (Qiagen). For the PCR, primers were designed to bind to the luciferase regions flanking the β -globin intron, giving rise to 2 PCR products that differ in lengths (2 bands on an agarose gel) depending on PNA uptake and splice correction. The primers used were forward (5'-ttg ata tgt gga ttt cga gtc gtc-3') and reverse (5'-tgt caa tca gag tgc ttt tgg cg-3'). The PCR program was as follows: 55°C for 35 minutes—1 cycle; 95°C for 15 minutes—1 cycle; 94°C for 0.5 minute, 55°C for 0.5 minutes, and 72°C for 0.5 minutes—33 cycles. The RT-PCR DNA products were analyzed on a 2% agarose gel with 1×TAE buffer and stained with ethidium bromide. Bands were visualized by Imagemaster^R VDS (Pharmacia Biotech) and analyzed by UNSCAN-IT software (Silk Scientific Corporation).

Fluorescence microscopy

Cells were grown for 24 hours in Laboratory-Tek 1 chambered Coverglass (Nunc; Thermo Fisher Scientific) before electrotransfer of fluorescein-labeled PNAs using the Cellaxess CX3 system. Electrotransfer was carried out with 0.1 μ M PNA by applying 10 pulses of 120 V for 25 ms. Cells were incubated at 37°C in 5% CO₂ atmosphere for 15 minutes after electrotransfer. Subsequently, the cells were washed once with phosphate-buffered saline and kept in a modified Hank's balanced salt solution for microscopy, as previously described (Kiryushko et al., 2006). Microscopy was carried out using a MultiProbe 2001 laser scanning confocal system equipped with an argon laser (excitation wavelength 488 nm, Radiance2000) connected to a Nikon eclipse TE200 microscope (oil immersion 60×1.4 NA objective; Nikon). The Lasersharpp 2000 software package (BioRad) was used for image acquisition and processing.

Statistics

All quantitative data are presented as mean $\pm$ standard deviation. For measuring statistically significant effects of

electroporation on luciferase readouts wherein more than 1 group has to be compared with the same control group (no electroporation), a 1-way ANOVA with a Dunnett's posttest was performed. For measuring statistically significant effects of PNA charge on electrotransferred cellular uptake, multiple *t*-tests with Bonferroni correction was performed. $P < 0.05$ was considered statistically significant.

Results

We varied pulse parameters with respect to Cellaxess and cuvette systems to identify optimal conditions, and this was combined with viability tests.

Electrotransfer of PNA using the Cellaxess CX3 system

A pulse of 120 V and varying pulse length. Standard parameters were applied for transfection of HeLa cells with the Cellaxess CX3 system (10 pulses of 120 V and a frequency of 1 Hz), and the pulse length was changed in steps in the range of 1–50 ms (Fig. 1). Figure 1a reports the resulting

FIG. 1. (a) Luciferase activity (RLU) in pLucHeLa cells exposed to electrotransfer of PNAs using Cellaxess CX3. PNA (23-): Negatively charged PNA3325. PNA (8+): Positively charged PNA2392. PNA (5+): Positively charged PNA3333. Neutral PNA: PNA2870. PNA concentrations were 1 μ M. Electroporation parameters: 10 pulses of 120 V and a frequency of 1 Hz. Results are presented as means $\pm$ SD ($n = 6$). * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$. For each PNA, the different electroporation parameters were tested against the control (no electroporation). (b) Reverse transcription (RT)–polymerase chain reaction (Cellaxess CX3). Analysis of the splice correction of luciferase mRNA on cells subjected to uncharged PNA2870 and electroporation with the Cellaxess CX3 system, using 10 pulses of 120 V and pulse lengths of 1–50 ms. Lane 1: no electroporation, no PNA; lane 2: 50 ms, no PNA; lane 3: no electroporation + PNA; lane 4: 1 ms + PNA; lane 5: 5 ms + PNA; lane 6: 10 ms + PNA; lane 7: 25 ms + PNA; lane 8: 50 ms + PNA. Experiments were independently repeated with similar results. PNA, peptide nucleic acid; RLU, relative light units; SD, standard deviation.

luciferase activity (relative light units) for the different pulse lengths and PNAs. As seen from the data, electroporation under these conditions had no significant effect on cellular uptake of the negatively charged PNA. In contrast, electroporation increased the cellular uptake for the positively charged (8+ and 5+) and charge neutral PNAs (Fig. 1a). For the (8+) positively charged PNA, a pulse length-dependent induction of luciferase activity was observed (Fig. 1a), whereas this was less clear for the PNA (5+). A maximum of 3–6-fold increase in luciferase activity above background (no electroporation) was obtained, which reached maximum at a pulse length of 5–10 ms. For the uncharged PNA, a maximum of about 14-fold increase in luciferase activity above background was obtained at a pulse length of 25 ms in a clearly pulse length-dependent fashion. Besides analyzing the effect of electroporation on cellular uptake within each of the 4 groups of PNAs, the effect of PNA charge on cellular uptake by electrotransfer was also statistically tested. The data show that uptake of the uncharged PNA was significantly higher when compared with the 3 other PNAs when a pulse length of 10 ms ($P < 0.01$), 25 ms ($P < 0.001$), or 50 ms ($P < 0.001$) was applied. This clearly demonstrates that the charge of the PNA has a decisive effect on electroporation-mediated cellular delivery. To further validate the data obtained in the luciferase assay in an absolute quantitative way, antisense activity was measured at the level of luciferase mRNA splice correction using RT-PCR. The results (Fig. 1b) confirm the enhancing effect of electroporation on cellular uptake of PNA, and using optimized parameters (120 V and pulse length of 25 ms), a maximum degree of splice correction of close to 25% was obtained, which would compare to full mRNA conversion given that only ~20% of the cells were indeed transfected in the geometry of electrodes employed by the Cellaxess system.

A pulse length of 10 ms and varying pulse amplitude. To investigate the effect of varying voltage, a pulse length of 10 ms was kept constant and 10 pulses at a frequency of 1 Hz with different volts ranging from 100 to 250 V were applied (Fig. 2). When pulses of 250 V were applied, the effect of the charge neutral PNA on cellular uptake (31-fold increase in luciferase activity above background) was shown to be statistically significantly higher ($P < 0.001$) than that of any of the other charged PNAs (not shown in the figure). These results confirm the conclusions from the pulse length variation experiments (Fig. 1) in terms of the different luciferase readouts between the differently charged PNAs (no charge > positive > negative).

Cell viability. Cell viability was also evaluated after electrotransfer with the Cellaxess CX3 system to investigate whether efficient PNA uptake was compatible with high cell survival (Supplementary Fig. S1a; Supplementary Data are available online at www.liebertonline.com/oli). The 2 parameters tested, 120 V for 50 ms and 250 V for 10 ms, are those with the highest applied voltage (250 V) and pulse length (50 ms) and the highest obtained cellular uptake of PNAs (Figs. 1 and 2). A reduction in cell viability was observed when applying a highest voltage of 250 V for 10 ms together with PNA irrespective of charge (Supplementary Fig. S1a). For all other conditions examined, no statistically significant reduction in cell viability compared with control was observed (Supplementary Fig. S1a).

FIG. 2. Luciferase activity (RLU) in pLucHeLa cells exposed to electrotransfer of PNAs using Cellaxess CX3. PNA (23-): Negatively charged PNA3325. PNA (8+): Positively charged PNA2392. PNA (5+): Positively charged PNA3333. Neutral PNA: PNA2870. PNA concentrations were 1 μ M. Electroporation parameters: 10 pulses of a pulse length of 10 ms and a frequency of 1 Hz. Results are presented as means $\pm$ SD ($n = 6$); * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $p < 0.001$. For each PNA, the different electroporation parameters were tested against the control (no electroporation).

Electrotransfer of PNA using the cuvette system

High voltage and low voltage parameters. The effect of electroporation on cellular uptake of PNA was also investigated with a cuvette electroporation system, using the same negatively charged, positively charged, and uncharged PNAs. High-voltage (HV) (1000 V/cm) and low-voltage (LV) (100 V/cm) pulses were applied to investigate the 2 standard electroporation settings that are frequently used for transfecting small or large charged molecules (such as DNA). Five LV pulses of 5 ms as well as 6 HV pulses of 0.1 ms were applied, respectively. As seen from the results presented in Fig. 3, the LV pulses had no significant effect on cellular uptake of any of the PNAs. In contrast, the HV pulses significantly increased the cellular uptake of the positively (but not uncharged or negatively) charged PNAs (Fig. 3). The largest effect was observed for the (8+) positively charged PNA [for which the effect was also statistically larger ($P < 0.001$) than that of any of the other PNAs], resulting in a 10-fold increase in luciferase activity over background (Fig. 3a). Again, the luciferase data were corroborated by RT-PCR data (Fig. 3b), confirming the enhancing effect of HV pulses on cellular uptake yielding a maximum of ~30% splice correction. Further, in electroporation-assisted cellular PNA delivery, PNA exhibited (as would be expected) a dose-response behavior in terms of increasing PNA concentration (0.3–1.3 μ M) (Fig. 4).

Cell viability. Finally, cell viability was tested after electrotransfer with the cuvette system, and no significant reduction in cell viability was observed when applying HV pulses (6 pulses of 1000 V/cm and 100 μ s) (Supplementary Fig. S1b). Surprisingly, when applying LV pulses (5 pulses of 100 V/cm and 5 ms), an apparent increase in cell viability was observed (Supplementary Fig. S1b). To investigate this phenomenon in more detail, a dose-response curve experiment

FIG. 3. (a) Luciferase activity (RLU) in pLucHeLa cells exposed to electrotransfer of PNAs using the cuvette system. PNA (23-): Negatively charged PNA3325. PNA (8+): Positively charged PNA2392. PNA (5+): Positively charged PNA3333. Neutral PNA: PNA2870. PNA concentrations were 1 μ M. Electroporation parameters: 5 pulses of 5 ms, LV, and a frequency of 1 Hz; 6 pulses of 0.1 ms, HV, and a frequency of 1 Hz. Results are presented as means $\pm$ SD ($n = 6$); * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$. For each PNA, the different electroporation parameters were tested against the control (no electroporation). (b) RT-polymerase chain reaction (cuvette system). Analysis of the splice correction of luciferase mRNA on cells subjected to positively charged PNA2392 and electroporation with the cuvette system. Lane 1: no electroporation + PNA; lane 2: LV pulses (5 pulses of 100 V/cm and 5 ms) + PNA; lane 3: HV pulses (6 pulses of 1000 V/cm and 0.1 ms) + PNA; lane 4: no electroporation, no PNA; lane 5: LV pulses (5 pulses of 100 V/cm and 5 ms), no PNA; lane 6: HV pulses (6 pulses of 1000 V/cm and 0.1 ms), no PNA. HV, high voltage; LV, low voltage.

was performed with pulses of 5 ms and increasing voltage from 12 to 200 V (Supplementary Fig. S1c). An increase in cell viability was indeed observed when applying LV pulses (30–100 V/cm), whereas application of pulses of more than 80 V resulted in a reduction in cell viability, with a larger reduction for higher voltages applied.

Fluorescence microscopy

In an attempt to investigate the intracellular uptake and localization of positively and uncharged PNAs after electrotransfer, confocal fluorescence microscopy was performed with fluorescein-labeled PNAs. The results are very clear concerning the uncharged PNA. In this case, electrotransfer-mediated cellular (and nuclear) uptake was observed using the Cellaxess CX3 system (Fig. 5), but not using the cuvette electrotransfer system (Fig. 6). This is fully in accordance with the antisense activity data (Figs. 1 and 3). The results concerning the positively charged PNA are less easily interpreted. First of all, in both cases, a strong adsorption of the

FIG. 4. Luciferase activity in pLucHeLa cells exposed to electrotransfer of PNAs at different concentrations, using the cuvette system and HV pulses. The positively charged PNA2392 was present in increasing concentrations (0.3–1.3 μ M). First column: No electroporation. Other columns: Electroporation of HV (1000 V/cm), 6 pulses of 100 μ s, and 1 Hz. EP, electroporation.

PNA to the cell surface is observed. However, upon electrotransfer, the cell images indicate that a slightly larger fraction of the PNA is internalized using the cuvette electrotransfer system (Fig. 6). The interpretation is clearly complicated by the fact that the major part of the positively charged PNA is slowly internalized via an endosomal pathway independent of electrotransfer. However, because of endosomal entrapment, the majority of the PNA internalized in this way is not functional in terms of antisense activity (Shiraishi et al., 2005; Shiraishi and Nielsen, 2006).

Discussion

Electrotransfer as a method for PNA delivery

In this study, the effect of electrotransfer on *in vitro* cellular uptake of PNAs with different charges was investigated, to explore new potential delivery strategies for PNAs on the road to possible future clinical use. Electrotransfer is a simple, transitory, and straightforward method for delivery of molecules, which may aid or substitute transfer, mediating modifications of the PNAs such as conjugation to delivery peptides or receptor-specific ligands. Electrotransfer may eliminate some of the problems (such as endosomal entrapment) encountered by exploiting other transport mechanisms across the membrane (Koppelhus and Nielsen, 2003; Lundin et al., 2006; Corey, 2007).

By focused delivery, using electrotransfer, the target cells can be selectively exposed to the molecules, and cells in other tissues may remain unaffected. Further, electrotransfer is widely used for *in vivo* gene electrotransfer, and it has become an acknowledged method in clinical use for enhancing delivery of chemotherapeutics. Currently, electroporators for clinical use are approved and have been proven efficient with few side effects in several clinical studies (Heller et al., 1998; Gothelf et al., 2003; Marty et al., 2006; Mir et al., 2006; Daud

FIG. 5. Confocal fluorescence microscopy after electrotransfer of PNA using the Cellaxess CX3 system. pLucHeLa cells exposed to electrotransfer of positively charged fluorescein-labeled PNA3266 or uncharged fluorescein-labeled PNA2817 using the Cellaxess CX3 system. Pseudocolor (Ps) and sectional (Sc) images were created by using the Laserssharp 2000 software package. EP, electroporation. PNA (8+): Positively charged PNA3266. PNA (n): Charge neutral PNA2817. *Left panel:* Charge neutral PNA and no electroporation. *Middle*

panel: Electroporation (parameters of 10 pulses of 120 V and 25 ms) and positively charged PNA. *Right panel:* Electroporation (parameters of 10 pulses of 120 V and 25 ms) and charge neutral PNA.

et al., 2008). Thus, the necessary technical setup for future electrotransfer of PNA in a clinical setting is ready once potential drug candidates have been identified.

Influence of electroporation configuration

It is remarkable that depending on the electroporation system used, optimal results are obtained with PNAs differing in charge. This might be due to the different physical setup of the 2 systems (Fig. 7). In the Cellaxess CX3 system, the PNA is flushed in for 60 seconds and electroporation is simultaneously applied during the last 10 seconds of flushing. In the cuvette system, the PNA is manually added to the cell suspension prior to electrotransfer. Therefore, in the cuvette system, the PNA molecules are present in the cell suspension before electrotransfer is performed and thus may more effectively adsorb to the cell membrane (Frantescu et al., 2005). This perhaps gives the advantage to the positively charged PNAs, having higher affinity for the negatively charged cell membrane, resulting in high concentrations of PNAs at the cell membrane prior to electroporation. In analogy, it has been shown that the efficiency of electrotransfer of oligonucleotides into lipid vesicles is enhanced by divalent cation-mediated adsorption to the surface of vesicles (Frantescu et al., 2005, 2006). Further, the cuvette system electroporates cells in suspension in the cuvettes, whereas the Cellaxess CX3 system electroporates adherent cells directly in 96 wells. It could be speculated whether charge neutral molecules have better access

to the adherent cells, in that they may pass better through the surrounding extracellular matrix than do charged molecules (Fig. 7). In this way, the charge of the molecules may influence the effect of electroporation on cellular uptake because of their different binding properties to cell membranes and extracellular matrix prior to electrotransfer. In addition, the cells used in suspension were trypsinized prior to electrotransfer, which could influence the properties of the cell membrane. These factors could all contribute to the differences in the results obtained with the 2 different electroporation systems.

Mechanism of electroporation-mediated uptake

According to electroporation theory, the largest area of permeabilization occurs at the side of the cell facing the positive electrode, and higher pulse amplitude will result in a greater area of the membrane being permeabilized. The permeabilization efficiency (per area) is greater on the side of the cell facing the negative electrode and is primarily controlled by the pulse duration (Gabriel and Teissie, 1997). After electroporation, diffusion alone is responsible for the cellular uptake of uncharged molecules, which mainly enter the cell at the side facing the positive electrode. For (larger) charged molecules such as DNA, electrophoretic forces play an important role in driving the negatively charged DNA through the membrane (Neumann et al., 1998; Bureau et al., 2000; Mir et al., 2005). Thus, the cellular electrotransfer of

FIG. 6. Confocal fluorescence microscopy after electrotransfer of PNA using the cuvette system. pLucHeLa cells exposed to electrotransfer of positively charged fluorescein-labeled PNA3266 or uncharged fluorescein-labeled PNA2817 using the cuvette system. Ps and Sc images were created using the Laserssharp 2000 software package. EP: Electroporation. PNA (8+): Positively charged PNA. PNA (n): Charge neutral PNA. *Left panel:* Positively charged PNA and no electroporation. *Middle*

panel: Electroporation (parameters of 6 pulses of 1000 V/cm and 0.1 ms) and positively charged PNA. *Right panel:* Electroporation (parameters of 6 pulses of 1000 V/cm and 0.1 ms) and charge neutral PNA.

FIG. 7. Schematic illustration of electrotransfer with the 2 electroporation systems: Cuvette system and Cellaxess CX3. *Left:* Electrotransfer of PNA with the cuvette system. The positively charged PNAs are likely adsorbed to the primarily negatively charged cell membrane prior to electrotransfer. *Right:* Electrotransfer of PNA with the Cellaxess CX3 system. The PNAs are being flushed out above cells growing in 96-well plates and have to pass through extracellular matrix to reach the cells. The time of flushing is 60 seconds, and electroporation is performed during the last 10 seconds of flushing.

positively charged PNAs would be expected to be aided by electrophoretic migration toward the cathode and negatively charged PNAs aided by electrophoretic migration toward the anode. The observation that positively charged PNAs are most efficiently delivered with the cuvette system (Fig. 3) is consistent with electroporation theory, in that the positively charged PNAs are expected to primarily enter the cells at the side facing the positive electrode, where the largest area of membrane permeabilization exists and in which direction the positively charged molecules are electrophoretically migrating toward the negative electrode. In the case of the Cellaxess system, it is therefore noteworthy that the noncharged PNA are by far most efficiently delivered. We speculate that when performing electrotransfer to adherent cells, the extracellular matrix may entrap charged molecules. The Cellaxess system uses short infusion time during the electric pulse, and therefore, neutral molecules may be more likely to arrive to the cell surface at the time when pulses occur.

Cell viability effects of PNA electrotransfer

A reduction in cell viability was observed only with the Cellaxess CX3 system and only when using electroporation parameters of 250 V for 10 ms together with PNA irrespective of charge (Supplementary Fig. S1a). However, it should be kept in mind that in this system, only around 20% of the cells in the well are exposed to electroporation, and therefore, a 100% decrease in viability of electroporation-affected cells would only result in 20% decrease in total viability. In the cuvette system, only the highest voltage pulses resulted in a slight reduction in cell viability (Supplementary Figs. S1b, c).

Conclusion

Although cellular delivery of PNA by electroporation has been exploited in a few studies (Shammas et al., 1999; Wang et al., 1999; Karras et al., 2001), the detailed mechanism of delivery as well as the effects of PNA charge modification and electroporation technology are largely unexplored. However, the present study, using a sensitive and quantitative HeLa cell assay with a functional readout, very clearly demonstrates that parameters such as PNA charge and the method and parameters of electroporation have dramatic influence on the efficiency of productive delivery. It is remarkable that changing the electroporation configuration from the micro-

titer plate format for monolayer cells to a more traditional 2-electrode cuvette configuration for cells in suspension decisively shifts the optimal PNA oligomer from a charge neutral to a positively charged PNA. Further, in neither case did a negatively charged (DNA-like) PNA show appreciable activity, although this PNA is active in the pLucHeLa cellular system in the nanomolar range when delivered via cationic liposomes (Shiraishi et al., 2008). These results would indicate that electroporative delivery of neutral gene therapeutics such as PNA or morpholino oligomers should be far more efficient than delivery of antisense oligonucleotides or siRNAs. Therefore, both the physicochemical properties of the active compound (drug) as well as the combination with the electroporation system will influence the outcome of electrotransfer-mediated cellular delivery, and these factors must be strategically and experimentally considered in biological experiments and, subsequently, clinical trials and use. As electrotransfer is presently in clinical use, *in vivo* studies on PNA electrotransfer strategies are warranted.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

Supplementary Materials and Methods

Supplementary Materials and Methods are available at *Oligonucleotide's* website (www.liebertonline.com/oli).

References

- BASU, S., and WICKSTROM, E. (1997). Synthesis and characterization of a peptide nucleic acid conjugated to a D-peptide analog of insulin-like growth factor 1 for increased cellular uptake. *Bioconjug. Chem.* **8**, 481–488.
- BENDIFALLAH, N., RASMUSSEN, F.W., ZACHAR, V., EBBESEN, P., NIELSEN, P.E., and KOPPELHUS, U. (2006). Evaluation of cell-penetrating peptides (CPPs) as vehicles for

- intracellular delivery of antisense peptide nucleic acid (PNA). *Bioconjug. Chem.* **17**, 750–758.
- BUREAU, M.F., GEHL, J., DELEUZE, V., MIR, L.M., and SCHERMAN, D. (2000). Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer. *Biochim. Biophys. Acta* **1474**, 353–359.
- CHRISTENSEN, L., FITZPATRICK, R., GILDEA, B., PETERSEN, K.H., HANSEN, H.F., KOCH, T., EGHOLM, M., BUCHARDT, O., NIELSEN, P.E., COULL, J., et al. (1995). Solid-phase synthesis of peptide nucleic acids. *J. Pept. Sci.* **1**, 175–183.
- COREY, D.R. (2007). Chemical modification: the key to clinical application of RNA interference? *J. Clin. Invest.* **117**, 3615–3622.
- DAUD, A.I., DECONTI, R.C., ANDREWS, S., URBAS, P., RIKER, A.I., SONDAK, V.K., MUNSTER, P.N., SULLIVAN, D.M., UGEN, K.E., MESSINA, J.L., and HELLER, R. (2008). Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. *J. Clin. Oncol.* **26**, 5896–5903.
- FRANDESCU, A., KAKORIN, S., TOENSING, K., and NEUMANN, E. (2005). Adsorption of DNA and electric fields decrease the rigidity of lipid vesicle membranes. *Phys. Chem. Chem. Phys.* **7**, 4126–4131.
- FRANDESCU, A., TONSING, K., and NEUMANN, E. (2006). Interfacial ternary complex DNA/Ca/lipids at anionic vesicle surfaces. *Bioelectrochemistry* **68**, 158–170.
- GABRIEL, B., and TEISSIE, J. (1997). Direct observation in the millisecond time range of fluorescent molecule asymmetrical interaction with the electroporeabilized cell membrane. *Biophys. J.* **73**, 2630–2637.
- GEHL, J. (2003). Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. *Acta Physiol. Scand.* **177**, 437–447.
- GEHL, J., SKOVSGAARD, T., and MIR, L.M. (1998). Enhancement of cytotoxicity by electroporeabilization: an improved method for screening drugs. *Anticancer Drugs* **9**, 319–325.
- GEHL, J., SORENSEN, T.H., NIELSEN, K., RASKMARK, P., NIELSEN, S.L., SKOVSGAARD, T., and MIR, L.M. (1999). *In vivo* electroporation of skeletal muscle: threshold, efficacy and relation to electric field distribution. *Biochim. Biophys. Acta* **1428**, 233–240.
- GOTHELF, A., MIR, L.M., and GEHL, J. (2003). Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation. *Cancer Treat. Rev.* **29**, 371–387.
- HAMILTON, S.E., SIMMONS, C.G., KATHIRIYA, D.S., and COREY, D.R. (1999). Cellular delivery of peptide nucleic acids and inhibition of human telomerase. *Chem. Biol.* **6**, 343–351.
- HANVEY, J.C., PEFFER, N.J., BISL, J.E., THOMSON, S.A., CADILLA, R., JOSEY, J.A., RICCA, D.J., HASSMAN, C.F., BONHAM, M.A., AU, K.G., et al. (1992). Antisense and antigenic properties of peptide nucleic acids. *Science* **258**, 1481–1485.
- HELLER, R., JAROSZESKI, M.J., REINTGEN, D.S., PULEO, C.A., DECONTI, R.C., GILBERT, R.A., and GLASS, L.F. (1998). Treatment of cutaneous and subcutaneous tumors with electrochemotherapy using intralesional bleomycin. *Cancer* **83**, 148–157.
- IVANOVA, G.D., ARZUMANOV, A., ABES, R., YIN, H., WOOD, M.J., LEBLEU, B., and GAIT, M.J. (2008). Improved cell-penetrating peptide-PNA conjugates for splicing redirection in HeLa cells and exon skipping in mdx mouse muscle. *Nucleic Acids Res.* **36**, 6418–6428.
- KANG, S.H., CHO, M.J., and KOLE, R. (1998). Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. *Biochemistry* **37**, 6235–6239.
- KARRAS, J.G., MAIER, M.A., LU, T., WATT, A., and MANOHARAN, M. (2001). Peptide nucleic acids are potent modulators of endogenous pre-mRNA splicing of the murine interleukin-5 receptor- α chain. *Biochemistry* **40**, 7853–7859.
- KIRYUSHKO, D., NOVITSKAYA, V., SOROKA, V., KLINGELHOFER, J., LUKANIDIN, E., BEREZIN, V., and BOCK, E. (2006). Molecular mechanisms of Ca(2+) signaling in neurons induced by the S100A4 protein. *Mol. Cell Biol.* **26**, 3625–3638.
- KLENCHIN, V.A., SUKHAREV, S.I., SEROV, S.M., CHERNOMORDIK, L.V., and CHIZMADZHEV, Y. (1991). Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis. *Biophys. J.* **60**, 804–811.
- KOPPELHUS, U., and NIELSEN, P.E. (2003). Cellular delivery of peptide nucleic acid (PNA). *Adv. Drug Deliv. Rev.* **55**, 267–280.
- LEBLEU, B., MOULTON, H.M., ABES, R., IVANOVA, G.D., ABES, S., STEIN, D.A., IVERSEN, P.L., ARZUMANOV, A.A., and GAIT, M.J. (2008). Cell penetrating peptide conjugates of steric block oligonucleotides. *Adv. Drug Deliv. Rev.* **60**, 517–529.
- LOHSE, J., NIELSEN, P.E., HARRIT, N., and DAHL, O. (1997). Fluorescein-conjugated lysine monomers for solid phase synthesis of fluorescent peptides and PNA oligomers. *Bioconjug. Chem.* **8**, 503–509.
- LUNDIN, K.E., GOOD, L., STROMBERG, R., GRASLUND, A., and SMITH, C.I. (2006). Biological activity and biotechnological aspects of peptide nucleic acid. *Adv. Genet.* **56**, 1–51.
- MAE, M., and LANGE, U. (2006). Cell-penetrating peptides as vectors for peptide, protein and oligonucleotide delivery. *Curr. Opin. Pharmacol.* **6**, 509–514.
- MARTY, M., SERSA, G., GARBAY, J.R., GEHL, J., COLLINS, C.G., SNOJ, M., BILLARD, V., GEERTSEN, P.F., LARKIN, J.O., MIKLAVCIC, D., PAVLOVIC, I., KOSIR, S.M.P., CE-MAZAR, M., MORSLI, N., SODEN, D.M., RUDOLF, Z., ROBERT, C., SULLIVAN, G.C., and MIR, L.M. (2006). Electrochemotherapy—an easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. *EJC Suppl.* **4**, 3–13.
- MIR, L.M., GEHL, J., SERSA, G., COLLINS, C.G., GARBAY, J.R., BILLARD, V., GEERTSEN, P.F., RUDOLF, Z., O'SULLIVAN, G.C., and MARTY, M. (2006). Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the Cliniporator (TM) by means of invasive or non-invasive electrodes. *EJC Suppl.* **4**, 14–25.
- MIR, L.M., MOLLER, P.H., ANDRE, F., and GEHL, J. (2005). Electric pulse-mediated gene delivery to various animal tissues. *Adv. Genet.* **54**, 83–114.
- NEUMANN, E., TOENSING, K., KAKORIN, S., BUDDE, P., and FREY, J. (1998). Mechanism of electroporative dye uptake by mouse B cells. *Biophys. J.* **74**, 98–108.
- NIELSEN, P.E., EGHOLM, M., BERG, R.H., and BUCHARDT, O. (1991). Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **254**, 1497–1500.
- OLOFSSON, J., LEVIN, M., STROMBERG, A., WEBER, S.G., RYTTSSEN, F., and ORWAR, O. (2005). Generation of focused electric field patterns at dielectric surfaces. *Anal. Chem.* **77**, 4667–4672.
- SAZANI, P., GEMIGNANI, F., KANG, S.H., MAIER, M.A., MANOHARAN, M., PERSMARK, M., BORTNER, D., and KOLE, R. (2002). Systemically delivered antisense oligomers

- upregulate gene expression in mouse tissues. *Nat. Biotechnol.* **20**, 1228–1233.
- SHAMMAS, M.A., SIMMONS, C.G., COREY, D.R., and SHMOOKLER REIS, R.J. (1999). Telomerase inhibition by peptide nucleic acids reverses “immortality” of transformed human cells. *Oncogene* **18**, 6191–6200.
- SHIRAISHI, T., and NIELSEN, P.E. (2006). Photochemically enhanced cellular delivery of cell penetrating peptide-PNA conjugates. *FEBS Lett.* **580**, 1451–1456.
- SHIRAISHI, T., HAMZAVI, R., and NIELSEN, P.E. (2008). Subnanomolar antisense activity of phosphonate-peptide nucleic acid (PNA) conjugates delivered by cationic lipids to HeLa cells. *Nucleic Acids Res.* **36**, 4424–4432.
- SHIRAISHI, T., PANKRATOVA, S., and NIELSEN, P.E. (2005). Calcium ions effectively enhance the effect of antisense peptide nucleic acids conjugated to cationic tat and oligoarginine peptides. *Chem. Biol.* **12**, 923–929.
- WANG, G., XU, X., PACE, B., DEAN, D.A., GLAZER, P.M., CHAN, P., GOODMAN, S.R., and SHOKOLENKO, I. (1999). Peptide nucleic acid (PNA) binding-mediated induction of human gamma-globin gene expression. *Nucleic Acids Res.* **27**, 2806–2813.
- WITTUNG, P., KAJANUS, J., EDWARDS, K., NIELSEN, P., NORDEN, B., and MALMSTROM, B.G. (1995). Phospholipid membrane permeability of peptide nucleic acid. *FEBS Lett.* **365**, 27–29.
- ZAHAROFF, D.A., BARR, R.C., LI, C.Y., and YUAN, F. (2002). Electromobility of plasmid DNA in tumor tissues during electric field-mediated gene delivery. *Gene Ther.* **9**, 1286–1290.
- ZHANG, X., SIMMONS, C.G., and COREY, D.R. (2001). Liver cell specific targeting of peptide nucleic acid oligomers. *Bioorg. Med. Chem. Lett.* **11**, 1269–1272.

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