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Formulation and Delivery of Splice-Correction Antisense Oligonucleotides by Amino Acid Modified Polyethylenimine

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Abstract: Splice-correcting phosphorothioate RNA antisense oligonucleotides with 2′-*O*-methyl modifications (ASO) are promising therapeutic agents for several disorders caused by aberrant splicing. However, their usefulness is hindered by the lack of efficient delivery. Unmodified 25 kDa polyethylenimine (PEI) has shown potential for plasmid delivery but seems to be less efficient for short nucleic acid sequences. Herein, we have evaluated several amino acid modified PEI molecules as carriers for ASO. By characterization of their properties, such as size, stability and transfection into mammalian cells, we have identified tyrosine-modified PEI (PEIY) as an efficient ASO delivery system. HeLa705 cells containing an aberrant luciferase gene, interrupted by a mutated β-globin intron, were used to assess the splice correction effectiveness mediated by the various modified PEI/ASO polyplexes. PEIY has a self-assembly nature, as opposed to the highly cationic parent polymer, which is relevant for the stability of the PEIY/ASO complexes. As a result, at an optimal ratio of 20:1 (+/−), the complexes that formed significantly corrected the splicing on both the mRNA and the protein levels. ASO formulated with PEIY enhanced luciferase activity up to 450-fold. This increase was three times higher than that produced by the commercially available transfection agent Lipofectamine. PEIY/ASO polyplexes resulted in at least 80% correct splicing of the transcript. Moreover, extremely low doses of ASO (0.025 μM) showed significant splice correction represented by 150-fold increase of luciferase activity and 47% mRNA correction. Our findings suggest key parameters for formulating active complexes and reveal a new platform that can be further developed for ASO *in vivo* targeting.

Keywords: Hydrophobic modification; polyethylenimine; splice correction; 2′-*O*-methyl phosphorothioate antisense oligonucleotides

Introduction

RNA-splicing is a process by which intronic sequences are removed from the primary transcript. Frequently a single pre-mRNA transcript can be alternatively spliced to produce multiple mRNA variants which in turn will be translated to different protein isoforms.[1] In fact, up to 70% of human genes undergo alternative splicing and, more importantly, up to 50% of human genetic diseases are known to arise from mutations that affect splicing.[2,3] Moreover, aberrations in alternative splicing have been observed in many cancer-

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related genes.[4,5] Therefore, optimization of drugs that can correct splicing mutations has recently become of great interest. Among ongoing splice correction trials, phosphorothioated oligonucleotides with 2′-*O*-methyl modifications are, particularly, found to be promising potential therapeutic agents for such diseases.[6] Numerous studies have already reported on the therapeutic potential of splice-switching oligonucleotides by targeting several diseases caused by aberrant splicing such as Duchenne muscular dystrophy, β thalassemia and atherosclerosis (reviewed in ref 7).

While new generations of oligonucleotides are more resistant to degradation, their therapeutic use is still limited because of their poor delivery.[6] The negatively charged phosphates in these oligonucleotides restrict their uptake into cells, and therefore the use of a positively charged carrier would be of potential help to their delivery. Polyethyleneimine (PEI) is a well-known polymer with high positive charge density. The electrostatic interaction between the positively charged amino acids in PEI and the negatively charged phosphate in nucleic acids leads to the subsequent condensation and formation of the polyplexes. Due to its cationic nature, PEI also binds to the negatively charged elements in the cell membrane thereby facilitating the endocytic uptake of the formed nucleotide−PEI complexes.[8,9] Moreover, PEI is characterized by its strong buffering effect over a broad range of pH; by exerting a "proton sponge" effect,[9] osmotic disruption of the vesicles may occur releasing the polyplex into the cytoplasm.[10,11]

Although showing great efficiency for delivery of plasmids,[12] PEI has been shown to be less efficient in delivery of short interfering RNA (siRNA)[13,14] and single-stranded antisense oligonucleotides (ASO).[15] This may be due to the weak interaction between the short oligonucleotides and PEI so that the polyplex cohesion cannot be maintained. Consequently, polyplexes may break rapidly upon contact with polyanions at the cell surface. Modification of PEI and/or its incorporation into other polymers have recently been tried for the formulation and delivery of such small oligonucleotides. One approach was by the use of hydrophobic molecules that can improve PEI−cell membrane interaction for delivery of siRNA. An example of these trials was the modification of branched PEI (25 kDa) by aliphatic lipids such as oleic and stearic acid, which resulted in better condensation and delivery of siRNA in B16 melanoma cells.[16] In another approach, negative charges were introduced into the polymer backbone to improve its efficiency and reduce the toxicity. An example of that was the introduction of propionic acid and succinic acid groups to the polymer structure.[17] A third approach was to reduce the surface charge of the polyplex by PEGylation, and this was used for delivery of antisense oligonucleotides.[18,19] One interesting trial using PEG-PEI copolymer for delivery of 2′-*O*-methyl splice-correcting oligonucleotides in cultured cells and in mdx mice has also been reported.[20,21]

In this study, by adding hydrophobic components to the PEI-molecule, the polymer was altered from a water-soluble into a self-assembly form. These modifications should confine higher stability to the short oligonucleotides' formulation. We have here used PEI modified with hydrophobic α-amino acids: leucine (L), phenylalanine (F), tryptophan (W) and tyrosine (Y). These amino acids constitute the cores of globular proteins and help maintain protein structures. Hence, we expected that modification of PEI by the addition of these types of amino acids would result in less toxic derivatives as compared to fatty acid modified PEI. Moreover, these PEI-derivatives were shown to retain the proton

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sponge and the nucleic acid binding properties of PEI amines since during the synthesis, a new cationic amine replaces the reacted one.[22] Previous studies with PEIY (PEI containing tyrosine) revealed this polymer as a promising siRNA carrier in mammalian cells.[22] siRNAs are significantly different from antisense RNA oligonucleotides, and herein, we set out to investigate if these dissimilarities could have major influences on the nucleic acid/polymer interaction and whether it would translate into activity. In addition, as opposed to siRNA, successful delivery of splice-switching ASO implies nuclear delivery. Essentially, we wanted to answer the question whether PEIY is as efficient for the delivery of 2′-*O*-methyl phosphorothioate-modified RNA-bases in single-stranded form as for the siRNA duplex. Moreover, we wanted to test the efficiency and compare the behavior of the other three modified derivatives for ASO delivery to what was previously reported for delivery of siRNA. This is the first proof for the effectiveness of PEIY as a delivery vehicle for splice-correcting oligonucleotides into mammalian cells that can be further developed for *in vivo* targeting.

Experimental Section

Materials. Active 2′-*O*-methyl phosphorothioate antisense oligonucleotide (ASO) (CCUCUUACCUCAGUUACA) used previously by Kang et al[23] was bought from Eurogentec S.A., Belgium. Unrelated, mismatched oligonucleotide (unASO) with the sequence CAGAGTTCTCAGGATGTA was used as a control.

PEI Derivatives. Commercially available 25 kDa branched PEI reference 40,872-7, batch 09529KD-466, was from Aldrich (St Quentin, France) and contains primary, secondary, and tertiary amines in a ratio of 1:1:1.[24] Modifications with the addition of the different amino acids were performed as previously described.[22] Briefly, the primary amines were allowed to react fully with the succinimidyl esters of butyloxycarbonyl (Boc)-protected amino acids. Boc groups were removed with trifluoroacetic acid followed by dialysis in aqueous HCl. This gave the desired products, with α-amino acid contents of 30% per ethylenimine (Figure 1). Tyrosine and tryptophane modified PEIs were also made with 20% substitution and compared to the ones with 30% modification. Hydrochloride salts of all the polymers are fully soluble in water and remained stable under long time periods. The polymers were stored as 0.5 M solutions at + 4 °C or at − 20 °C when for longer periods.

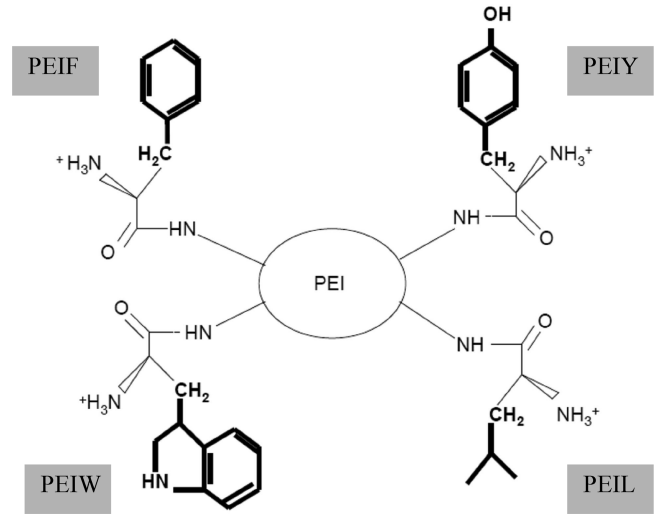

Figure 1. Structures of the different modified PEIs (L = leucine, W = tryptophan, Y = tyrosine and F = phenylalanine).

PEIY/ASO Complex Formation. Formulations of ASO nanoparticles were prepared by equivolumetric mixing of the oligonucleotide at the desired concentration with the PEI derivative at various concentrations corresponding to the different charge ratios (NH_3^+/PO_4^-) ranging from 5:1 to 40:1. Prior to mixing, ASO and PEI derivatives were first incubated for five minutes in serum-free Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Germany). ASO complexes were incubated at room temperature for 30 min before a 5-fold dilution into DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Unless stated otherwise, all protocols for ASO complexes used this same preparation method.

Polyplex Stability: Polyanion Competition Assay. The relative stability of polyplexes was evaluated by measuring ASO released from the polyplexes in the presence of a competing polyanion.[25] Polyplexes of ASO with PEI or its amino acid modified derivatives were prepared in 20:1 ratio. Samples containing 30 pmol of ASO corresponding to 200 ng were incubated for 15 min at 37 °C in the presence of heparin sodium (Sigma-Aldrich, Germany) over a range of concentrations. Samples were analyzed on 1.5% agarose gels in 1× TAE buffer and visualized by staining with sybergreen II (Invitrogen, Molecular Probes). Gels were documented using the Fluor-S system with a cooled CCD camera (BioRad, USA) and analyzed with the Quantity One software (BioRad). At a given heparin concentration, the percentage of the ASO released from the formulation was calculated by normalizing against the ASO band from a sample containing ASO alone loaded on the same gel.

Particle Size Measurements. Hydrodynamic mean diameter of the PEI/ASO particles was determined by dynamic light scattering (DLS) studies using a Zetasizer Nano ZS

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apparatus (Malvern Instruments, United Kingdom). Particle formulation followed the same protocol as for *in vitro* transfections, with a final oligonucleotide concentration of 0.1 μM, and, accordingly, dilution was performed in 10% fetal bovine serum (FBS) supplemented medium. In order to avoid light absorbance, experiments were performed in Opti-MEM medium (Invitrogen). Specifications of measurements inherent to the medium used followed those previously reported.[22] All DLS results were based on three measurements from three independent samples. All data were converted to "relative by intensity" plots from where the mean hydrodynamic diameter was derived.

Toxicological Effects of PEIY and PEIY/ASO Particles. Effects of PEIY and PEIY/ASO particles on the proliferation of cells were tested using two cell lines: HeLa705 (human cervical cancer cells, kindly provided by Professor Kole[23]) and BHK (baby hamster kidney cells, obtained from ATCC). The new xCELLigence system (Roche, Germany) was used to monitor the cell proliferation and viability for several time points, while the conventional WST-1 assay (Roche, Germany) was used to confirm the results.

The xCELLigence system assay was performed according to the manufacturer's protocol with some modifications. 1.5×10^4 and 3×10^5 BHK and HeLa705 cells, respectively, were seeded in the E-plate and cultured for approximately 20 h at 37 °C, 5% CO_2/air, before the addition of PEIY or PEIY/ASO. Changes in the cell status were monitored and quantified by detecting sensor electrical impedance every 15 min during 45 h. Cell index (CI) was derived to represent the cell proliferation based on the measured electrical impedance.[26] The presented "normalized cell index" results from the ratio between the cell index at the time point immediately before the addition of the PEIY or PEIY/ASO and that at the time point 0 h.

WST-1 assay followed the protocol provided by the manufacturer with small modifications. Briefly, BHK and HeLa705 cells were seeded at a density of 7×10^3 and 1×10^4 cells, respectively, per well in 96-well plates and maintained in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) 24 h before transfection. Prior to sample addition the cell medium was replaced by 100 μL of fresh DMEM supplemented with 10% FBS. A volume of 20 μL of the different concentrations of PEIY or PEIY/ASO, ranging from 0.09 to 0.9 mM, was then added to the cells. After 4 h incubation at 37 °C, 50 μL of DMEM supplemented with 10% FBS was added to each well. Cells were further incubated for 21 h. The number of surviving cells was determined by the WST-8 assay. Cell proliferation was expressed as the ratio of the A_{450} of treated cells to that of the untreated cells.

Cell Culture and *in Vitro* Transfections. One day prior to transfection, HeLa705 cells were seeded at a density of 50 000 cells per well in 24-well plates, at a final volume of 500 μL. The final concentration of ASO ranged from 0.025 to 0.1 μM, and the amount of PEIY used varied accordingly. PEIY/ASO complexes were prepared in 100 μL volume, as described above, using different amounts to give charge ratios between 2:1 and 40:1. Cells were harvested for analysis 24 h post-transfection. The same protocol was applied to all PEI derivatives. Transfections with Lipofectamine and Oligofectamine (Invitrogen) were used as a control and were performed according to the protocol provided by the manufacturer. An unrelated 2′-*O*-methyl phosphorothioate oligonucleotide (unASO) was used as a control for the targeting oligonucleotide.

Splice Correction Studies: Protein Activity and mRNA Levels. For analysis of the luciferase activity, cells were harvested in Reporter lysis buffer (Promega, USA). The protein's activity was assessed using luciferase reagent (Promega) and monitored in Fluostar optima (BMG LABtech, Germany). Total protein was measured using the Micro BCA protein assay kit (Thermo Scientific-Pierce Protein Research Products) and according to the manufacturer's protocol. For RT-PCR, cells were trypsinized and total RNA was isolated from the cell pellets using the RNeasy plus kit (QIAGEN, Sweden). The quality of RNA was verified by agarose gel elctrophoresis. Three nanograms of RNA was used in each RT-PCR reaction in which the total volume was 20 μL using the ONE STEP RT-PCR kit (QIAGEN). The primers had the following sequences: Fwd-TTGATATGTGGATTTCGAGTCGTC; Rev-TGTCAATCAGAGTGCTT-TTGGCG. The program for the RT-PCR was as follows: 55 °C, 35 min, then 95 °C, 15 min, for reverse transcription step directly followed by the PCR (94 °C, 30 s, then 55 °C, 30 s, then 72 °C, 30 s) for 30 cycles and finally 72 °C, 10 min, for final extension. The PCR products were analyzed in a 2% agarose gel in 1× TBE buffer and visualized by SYBR Gold (Invitrogen, Molecular Probes) staining. Gels were documented using the Fluor-S system with a cooled CCD camera (BioRad) and analyzed with the Quantity One software (BioRad).

Statistics. Data are expressed as mean ± standard deviation. Statistical analyses were performed using Student's *t*-test for comparison of means. A probability of less than 0.05 was considered to be statistically significant.

Results

Characterization of Different Modified-PEI/ASO Complexes: Formation, Size and Stability. The particles' size has been shown to be of great importance for a successful delivery.[27] DLS studies were conducted to assess the hydrodynamic mean diameter of the complexes resulting from the interaction of various PEI derivatives with the oligonucleotide. Due to the presence of proteins and protein-related aggregates, a population of particles with an average

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Table 1. Average Size of the Oligonucleotide Nanoparticles[a]

formulation	av diameter (nm)
PEIY/oligo 5:1	ND
PEIY/oligo 10:1	165.2 ± 52.6
PEIY/oligo 20:1	133.5 ± 18.7
PEIY/oligo 30:1	135.7 ± 30.4
PEIY/oligo 40:1	77.1 ± 15.6*

[a] Splicing correction ASO, at a concentration of 0.1 μM, were formulated with PEIY at different N/P ratios, according to the protocol for *in vitro* transfections. Characterization of the particles' size was done by dynamic light scattering. Each average size represents a mean of at least 3 samples. Mean diameters were compared for significance according to Student's *t*-test. Differences in the average diameter of particles formulated at ratio 40:1 were found significant when compared to the other ratios, $*p < 0.05$.

Table 2. Comparison between ASO Particles Formed by Different PEI Derivatives at a Ratio of 20:1[a]

	av diameter (nm)
PEIY	196.4 ± 44.0
PEIY/oligo	133.5 ± 18.7
PEIW	301.6 ± 48.0
PEIW/oligo	241.9 ± 46.3
PEIF	ND
PEIF/oligo	216.5 ± 62.6

[a] Formulation followed the protocol used for *in vitro* transfections, and freshly formed nanoparticles were characterized by dynamic light scattering studies. Each average size represents a mean of at least 2 samples. Significant differences were found between the mean diameters of each polymer and its ASO formulation ($*p < 0.05$). Significant differences were also found between the mean diameters of PEIY/ASO and PEIW/ASO and PEIF/ASO ($*p < 0.05$) but not between the PEIW/ASO and PEIF/ASO nanoparticles' mean diameters.

size of 40 nm was observed while analyzing OptiMEM supplemented with 10% FBS (Supplementary Figure 1 in the Supporting Information). This represents the background population during the assessment of the oligonucleotide complexes, and any disturbances in this distribution curve or the materialization of a new population are attributed to the oligonucleotide nanoparticles (Supplementary Figure 1 in the Supporting Information). As previously suggested,[22] PEIY and PEIW are self-assembling systems resulting in the formation of nanoparticles in the absence of oligonucleotides (Tables 1 and 2). These nanoparticles can be seen as a clear distinctive population from that of the background, found in the FBS supplemented medium. Addition of ASO induces significant differences in the particle size resulting in smaller size averages (compare PEIY and PEIW with the corresponding formulated particles at the ratio of 20:1). As shown in Table 1 the mean diameter of the PEIY/ASO complexes decreased with increasing charge ratios (NH_3^+/PO_4^-) of the complexes, although the difference is only significant for the highest ratio tested. Most probably, this phenomenon is a consequence of increased interaction between PEIY and the oligonucleotide resulting in a tighter binding. PEIW/ASO and PEIF/ASO nanoparticles, formulated at a 20:1 ratio, did also result in the formation of a distinct population, having an average diameter of 242 and 217 nm, respectively. These were found to be significantly larger as compared to PEIY/ASO, correlating to the difference in activity. On the contrary, when PEI and PEIL were added to ASO in the same ratios as PEIY, -F and -W, no disturbance occurred in the background population, despite the fact that they formed particles in absence of serum (data not shown). This suggests a dissociation of the complexes by ionic competition of the proteins present in the FBS.

To verify the formation of PEIY/ASO containing particles PEIY was first added to ASO, in different concentrations, and the formulations were evaluated in an electrophoresis gel. Due to the shielding effect of the PEIY, the oligonucleotide complexes are not visible on the agarose gel, a fact that was utilized to study the stability differences between the polymers. Owing to charge competition, addition of increasing concentrations of negatively charged heparin to the formulation induced the release of the ASO from its complexes with the polymers, making it available to SYBR Green II. This dye is well-known to specifically stain single-stranded oligonucleotides. Polyplexes with PEI and PEIL showed fast release of ASO while those with PEIW and PEIF were exceptionally stable showing significantly slower release rate of the oligonucleotide when incubated with heparin as competing anion. Interestingly, PEIY formed moderately stable ASO polyplexes here translated into a slower release rate than that of PEI and PEIL and a higher plateau than that resulting from PEIW and PEIF (Figure 2A). The heparin concentration required to release 50% (IC_{50}) of ASO from its complexes with PEIF and PEIW was found to be 2515 (±172) and 2910 (±127) μg/mL, respectively, while for PEI and PEIL-containing polyplexes this value was 514 (±52) μg/mL and 503 (±33), respectively (Figure 2B). IC_{50} value for PEIY/ASO polyplexes was moderate (936 (±150) μg/mL) and significantly different from the other compounds.

The Effect of PEIY/ASO Complexes on Cell Proliferation. Cytotoxicity can be a limiting factor for the development of any drug carrier and, thus, is crucial to be considered at early stages. To better evaluate the effect of PEIY/ASO complexes on cell proliferation, cytotoxicity studies were conducted in two different cell lines, BHK and HeLa705. Cytotoxicity was, also assessed by two different methods (Figure 3), and both revealed no significant difference between cells treated with formulated or unformulated PEIY. BHK cells were more sensitive than HeLa705, resulting in a lower IC_{50} (half-maximal inhibitory concentration) value when calculated by the xCELLigence system (1.14×10^{-4} M versus 2.24×10^{-4} M, for HeLa705 cells). The high growth rate of the BHK cells could possibly contribute to the higher sensitivity as compared to HeLa705 cells, resulting in the difference between the two cell lines treated with the various PEIY concentrations. Similar cell-

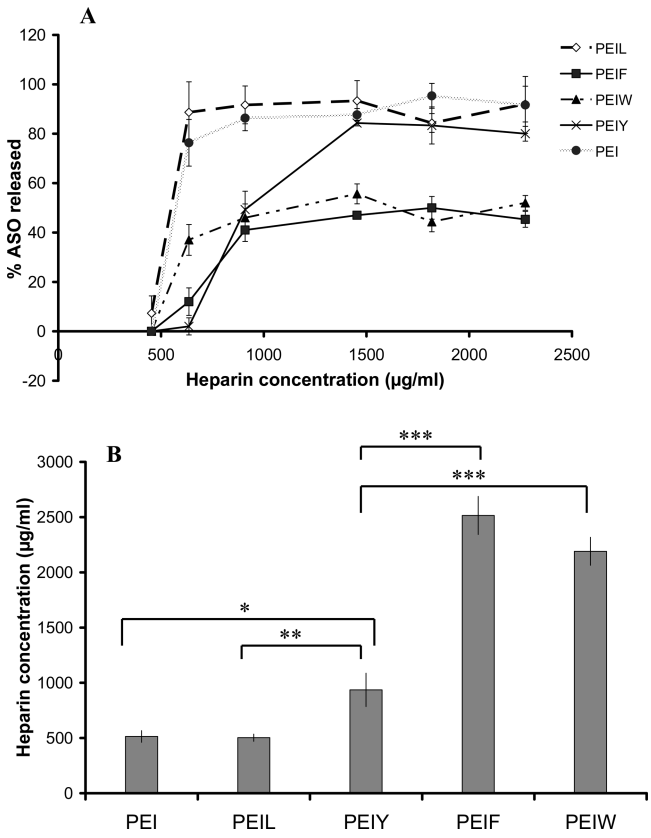

Figure 2. Evaluation of polyplexes stability by heparin competition assay. (A) Destabilization of ASO polyplex with PEI or its amino acid modified derivatives through incubation with increasing concentrations of heparin at 37 °C for 15 min. Samples were run on 1.5% agarose gel and visualized by SYBR Green II staining. Intensities of bands were calculated, and percentages of released ASO to the total ASO amount were plotted. (B) Heparin concentration required to release 50% of ASO from the polyplexes (IC_{50}) was calculated for formulations with PEI and its modified derivatives. Difference in the IC_{50} of particles formulated with PEIY was found to be significant compared to PEIF and PEIW (***$p < 0.001$) and was also found to be significant when compared to PEI (*$p < 0.05$) and to PEIL (**$p < 0.01$).

dependent cytoxicity has been previously reported[28] underlining the importance of the target cells when analyzing any application of potential therapeutic agents. By looking at Figure 3B, where it is possible to follow the proliferation of the cells over time, BHK cells seem to tolerate PEIY up to the concentration of 0.04 mM whereas HeLa705 cells tolerate twice as much (0.09 mM). In Figure 3B, showing the HeLa705 cell line, it is possible to find higher, although not significant, values of normalized cell index for the PEIY concentrations of 0.09 and 0.18 mM, as compared to untreated cells. These are most probably due to a small discrepancy in number of cells seeded, which was not initially detected by the instrument.

Evaluation of Oligonucleotide Delivery by PEIY: Studies on Protein Activity and mRNA Levels. Effect of Different Charge Ratios. HeLa705 are cells stably transfected with a luciferase gene in which the coding region is interrupted by a mutated β-globin intron.[23] The mutation results in the activation of a cryptic splice site causing defective splicing and a dysfunctional protein. Blocking of the mutation with the ASO corrects splicing, and thus, the translated mRNA will correspond to the active form of the protein.[23] To study the efficiency of PEIY-mediated delivery of ASOs, we transfected HeLa705 cells with PEIY/ASO formulations at different charge ratios from 2:1 to 40:1 using a constant final concentration of 0.05 μM of the ASO. On the protein level, the fold increase in luciferase activity at the 10:1 ratio was about 170, a figure similar to the one achieved using the lipid-based transfection reagent Lipofectamine (Figure 4, upper panel). Higher ratios of the PEIY gave higher fold increases in luciferase activity until reaching about 450-fold increases using the 20:1 ratio with no considerable effect on the total amount of protein in the cell cultures (5.8 μg versus 6.5 μg in untreated cells). Further increase in ratios did not yield significantly higher enzyme activity. On the mRNA level, ASO formulated with PEIY caused as high, or higher, correction levels as was achieved with Lipofectamine and much higher than when using Oligofectamine or the unmodified branched 25 kDa PEI (Figure 4 lower panel). In average, from three different experiments, ASO formulated with PEIY in a ratio of 10:1 (+/−) resulted in 63% (±3) and the 20:1 ratio gave 82% (±4) correction, while the control Lipofectamine formulations gave 71% (±5) correction at the RNA level. Further increases in PEIY/ASO charge ratios showed slightly higher correction until reaching about 85% (±1) using the 40:1 ratio.

PEIY Formulation Allows Efficient Delivery Also at Lower ASO Concentrations. ASO was then transfected at different final concentrations using PEIY in the ratio 20:1 and compared to ASO formulated with Lipofectamine. As expected, the increase in luciferase activity compared to untreated HeLa705 cells was found to be dose-dependent. Interestingly, PEIY in 20:1 ratio was more potent than Lipofectamine over the whole concentration range tested. The increase in luciferase using 0.025 μM of the splice-correcting oligonucleotide was about 130-fold compared to untreated cells (Figure 5, upper panel). Lipofectamine, on the other hand, gave only a 20-fold increase at the same concentration. Similarly, on the mRNA level, the correction of splicing increased in a dose-dependent manner with PEIY displaying higher efficiency than Lipofectamine over all concentrations (Figure 5, lower panel). Even at the lowest dose tested (0.025 μM) we could get about 47% splice correction. When the mismatched unrelated oligonucleotide (unASO) was transfected at the highest concentration used for the specific ASO (0.1 μM), it did not markedly influence either the protein or the RNA level.

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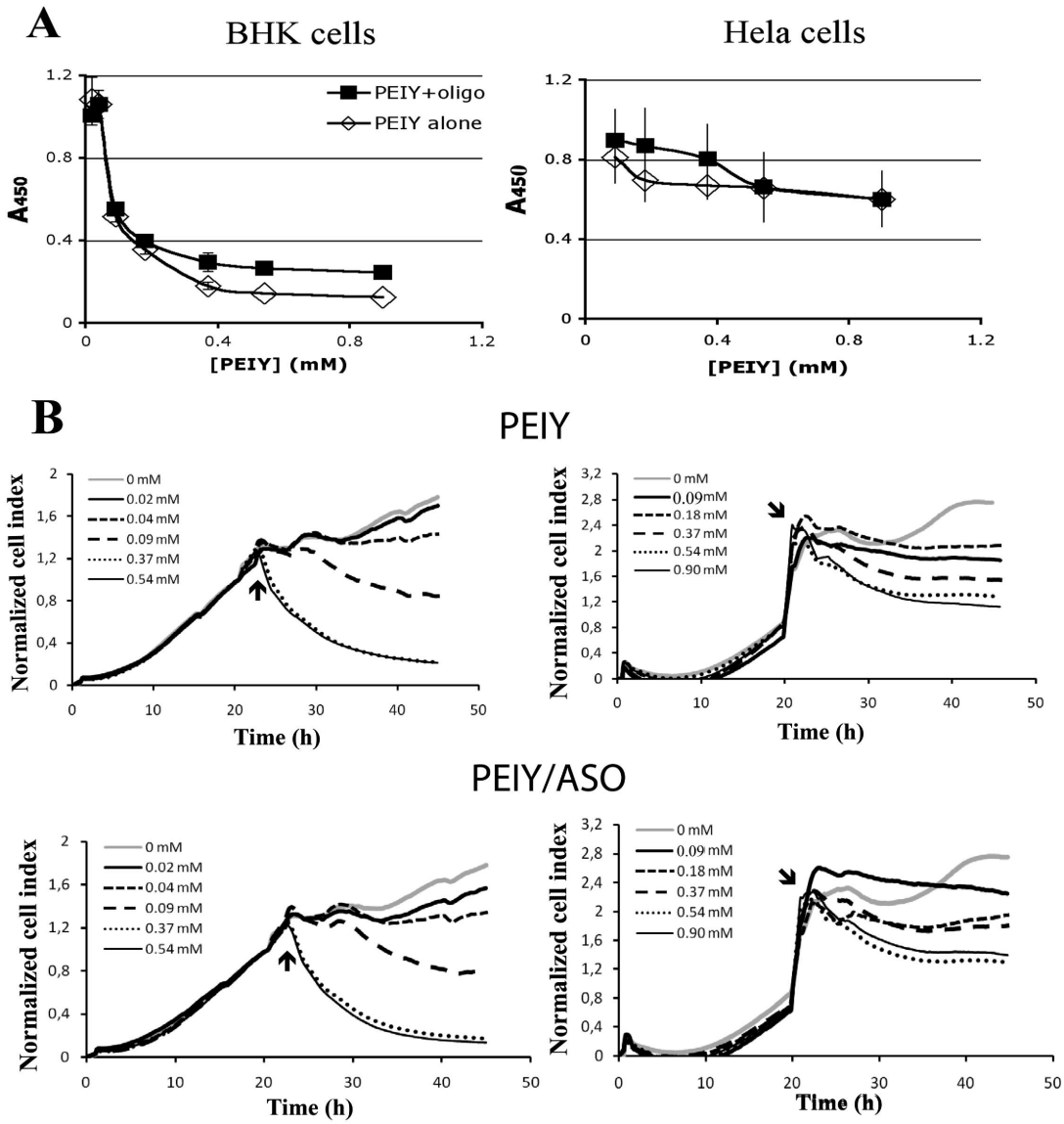

Figure 3. *In vitro* evaluation of the toxicity of PEIY. The cytotoxic properties of PEIY were assessed by (A) WST-8 reagent assay and (B) xCELLigence system for *in vitro* conditions with BHK (on the left) and HeLa705 (on the right) cells. Different concentrations of PEIY alone or formulated with short single oligonucleotides, at the (+/−) ratio of 20:1, were added to the cells. (A) For WST-8 reagent (Roche) assay the absorbance was measured at 450 λ and is here shown as the relative value of treated to untreated cells. Each point represents a mean ± SD of two independent experiments. (B) PEIY was added to the cells 20 h postseeding, and the effect of the formulations on cell proliferation was followed over 45 h with the xCELLigence system (Roche). The cell index (CI) represents the cell proliferation and was here normalized for a better distinction and comparison between the different concentrations. Each concentration is here shown as an average of four replicates. The figure represents one of the three independent experiments that were conducted. The arrow points out the addition time of PEIY, alone or with oligo.

Effect of Other Amino Acid Modifications and the Content of Tyrosine in PEIY on ASO Delivery. Finally we investigated whether any of the other amino acid modifications, leucine, phenylalanine and tryptophane, behaved differently in the delivery of single-stranded 2′-*O*-methyl oligonucleotides as compared to what was earlier reported for double-stranded siRNA. These polymers were used to formulate the ASO at a single charge ratio of 20:1, as this was found to be the most optimal for the PEIY-modified polymer. As can be seen in Figure 6, these polymers were all found to be less effective than both PEIY and Lipofectamine when used to transfect HeLa705 cells. When the tyrosine content of PEIY (30% tyrosine/ethylenimine unit) was decreased to 20% ($PEIY_{20}$), its efficiency in ASO delivery was significantly decreased as shown in both protein and mRNA levels (Figure 6 upper and lower panels). This indicates that the efficacy of PEIY is also dependent on the degree of tyrosine modification, indicating the importance of keeping a certain hydrophobic/hydrophilic balance for the effectiveness of this polymer. We also investigated whether changing the amino acid content to 20% in the ineffective derivative P̈EIẄ would improve its delivery efficiency. When

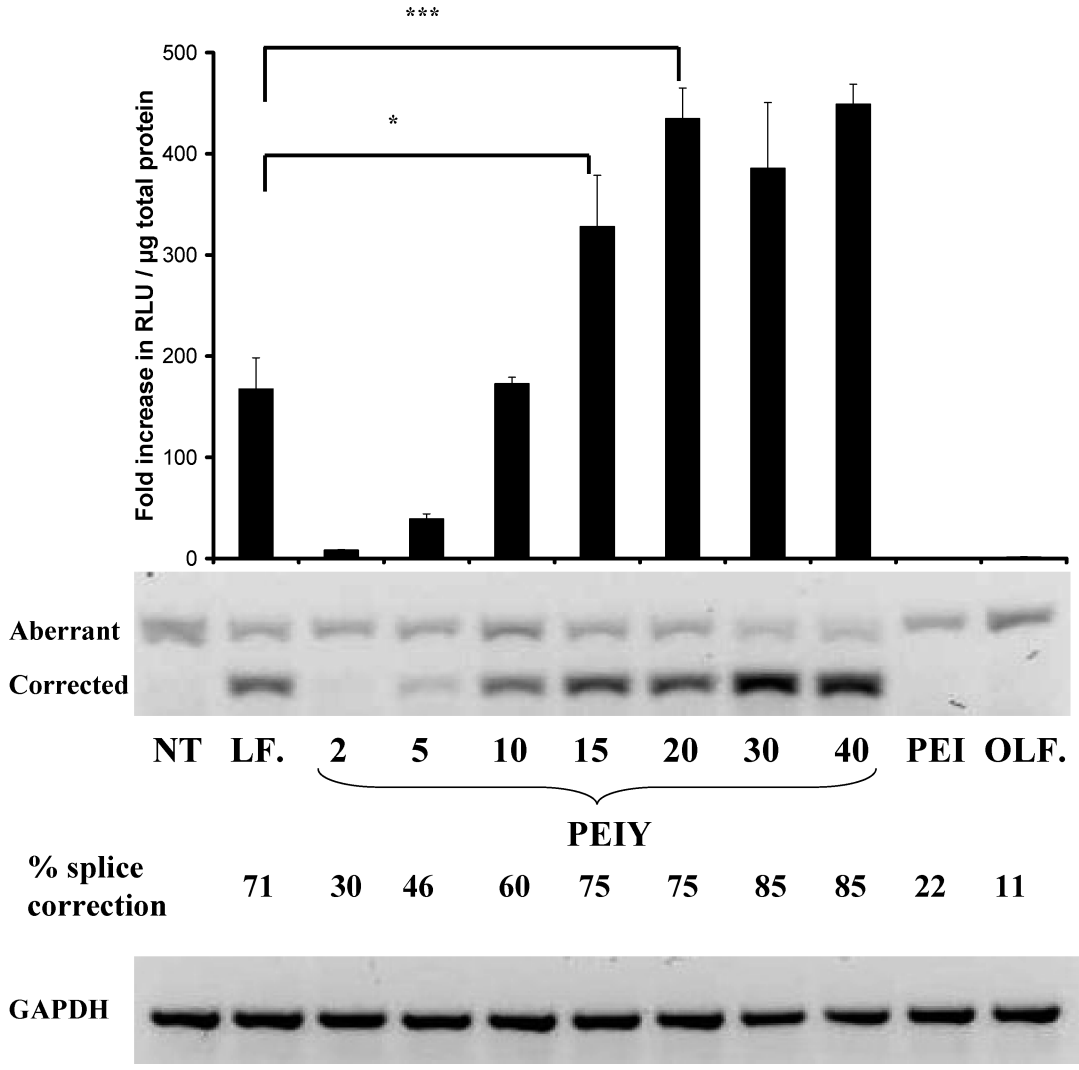

Figure 4. Levels of splice correction by ASO/PEIY complexes. ASO was formulated with PEIY at a final concentration of 0.05 μM, in different charge ratios (+/−) from 2:1 to 40:1 (2 to 40) and compared to Lipofectamine (LF.), Oligofectamine (OLF.) and parent PEI 25 kDa (PEI). HeLa705 cells were harvested for analysis 24 h post-transfection. Upper panel: Luciferase activity was quantified by relative luminescence units (RLU) and is here shown as a relative increase to that of untreated cells (NT). Each bar represents a mean ± SD of three independent experiments. Differences in fold increase between ASO formulations with PEIY in 15:1 and 20:1 and with Lipofectamine were significant (*$p < 0.05$ and ***$p < 0.0001$ respectively). Lower panel: RT-PCR for the RNA extracted from cells after transfection with the same formulations as in the upper panel. The levels of corrected mRNA were analyzed in 2% agarose gel and quantified using the Quantity One software. Splice correction was calculated as "the percentage of the corrected luciferase band to the sum of corrected and aberrant bands". The figure is representative of three independent experiments. Housekeeping GAPDH mRNA is shown as a control.

$PEIW_{20}$ was tested for transfection of ASO, it was still inefficient both on the protein and RNA levels (data not shown), highlighting the difference between double-stranded RNA and single-stranded chemically modified RNA oligonucleotides.

Discussion

Antisense oligonucleotides, as well as siRNAs, have been shown to hold great potential as therapeutic agents for several genetic and viral diseases. However, both approaches are hampered by many delivery barriers; their lack of protection to degradation and of targeting capability.[29] Recently developed ASOs with phosphorothioate and 2′-*O*-methyl modifications were shown to be more resistant to degradation and have been proven efficient upon intravenous injection.[30,31] However, the lack of a targeted carrier system means the usage of large amounts of material, turning this treatment

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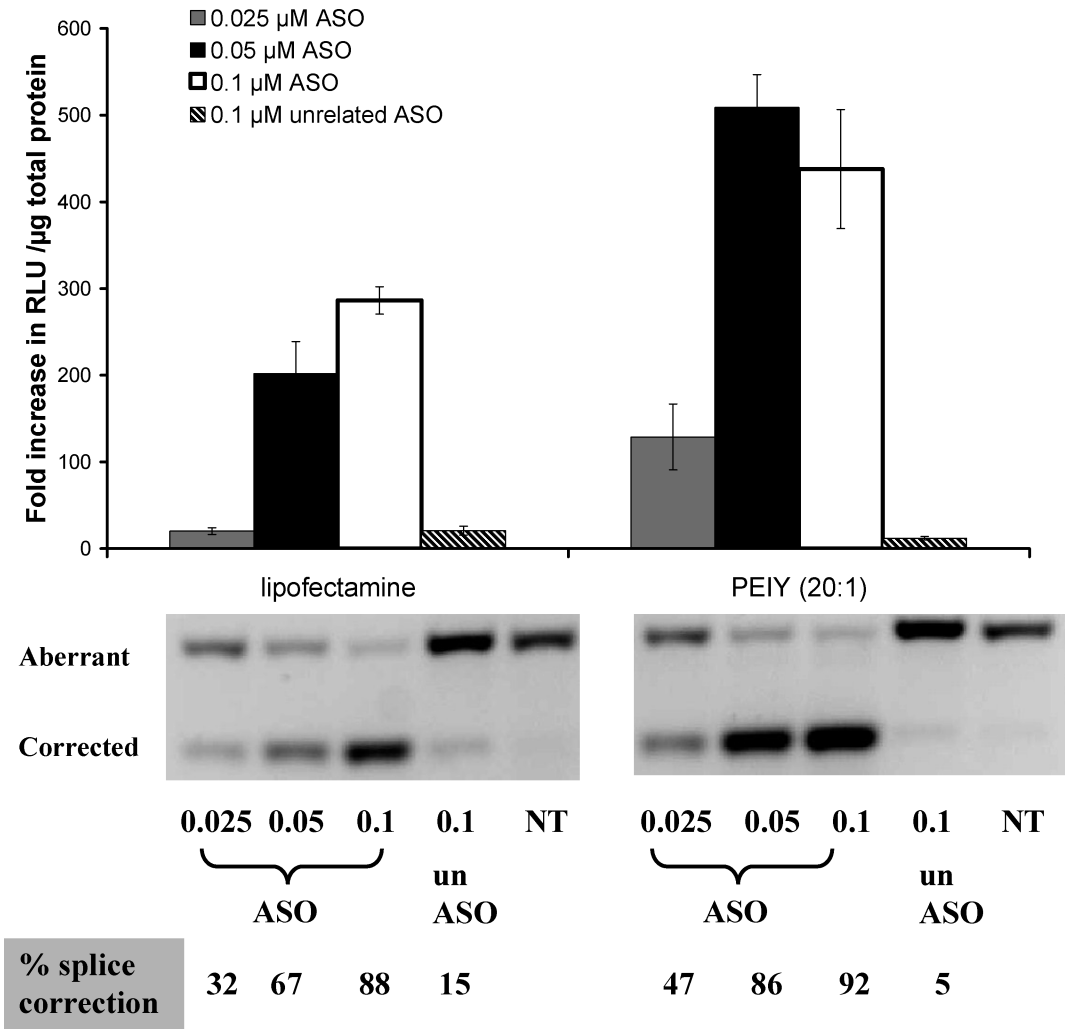

Figure 5. Effect of ASO concentration on the splice correction. HeLa705 cells were transfected with different concentrations of ASO (0.025−0.1 μM) formulated with either PEIY at the charge ratio 20:1 (+/−) or Lipofectamine. The control oligonucleotide (unASO) was also transfected the same way using the highest concentration tested (0.1 μM). Splice correction was evaluated by measuring the levels of protein activity (upper panel) and corrected mRNA (lower panel). Upper panel: Luciferase activity shown as fold-increase in RLU relative to that of untreated cells. Each bar represents a mean ± SD of three independent experiments. Lower panel: RT-PCR for the RNA extracted from cells after transfection with the same formulations as in upper panel. Corrected mRNA levels were quantified in 2% agarose gel using the Quantity One software. % splice correction was calculated as previously described in Figure 4. The figure is representative of three independent experiments. Note: The final concentrations of PEIY in the well, when complexed with ASO at the 1:20 ratio, are as follows: for 0.025 μM ASO, 0.045 mM PEIY; for 0.05 μM ASO, 0.09 mM PEIY; and for 0.1 μM ASO, 0.18 mM PEIY.

into a highly expensive approach in clinical settings. For the first time, tyrosine-modified PEI (PEIY) was herein established as a promising delivery system for such oligonucleotides. In an initial evaluation stage, for *in vitro* transfections, oligonucleotides formulated with PEIY at an optimal ratio (≥20:1 (+/−)) resulted in at least 80% splice correction of the defective transcript. This ratio was significantly higher than the one achieved by Lipofectamine at the same oligonucleotide concentration. This is higher than the correction on mRNA level that was previously reported using other delivery systems or oligonucleotide analogues like PNA.[32−35] The correction on the protein level was also remarkable (450-fold) and beyond what has been reported by many studies involving the delivery of similar oligo-

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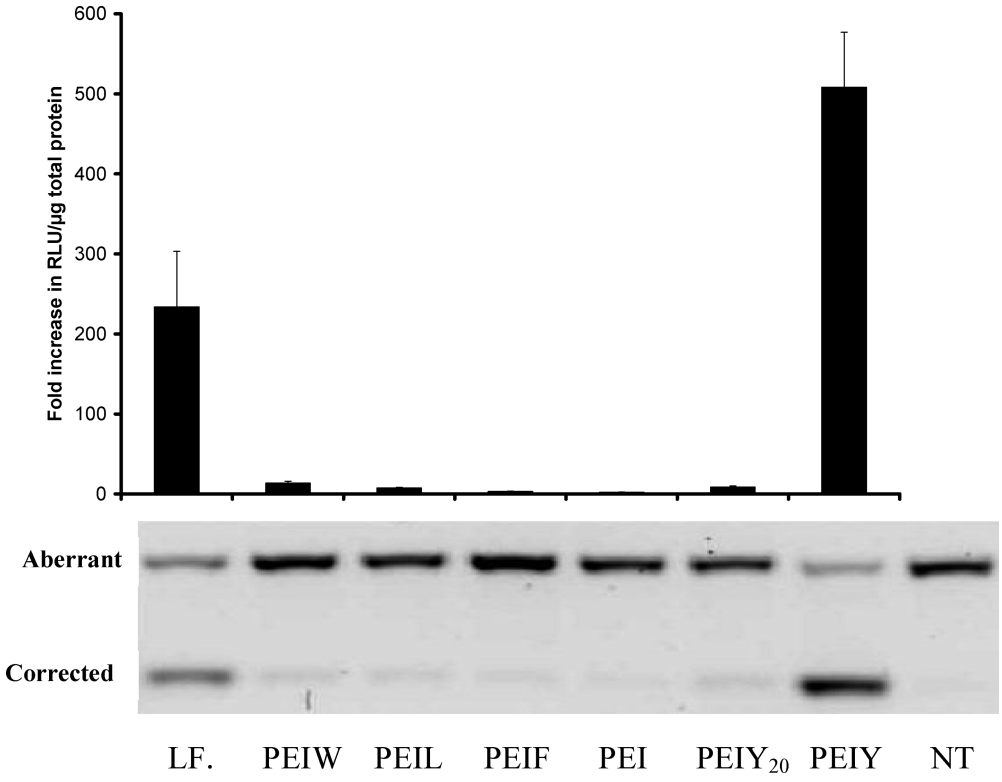

Figure 6. Effect of other amino acid modifications and of the tyrosine content in PEIY on the efficiency of the polyplex delivery of ASO. HeLa705 cells were transfected with ASO at final concentration 0.05 μM using Lipofectamine (LF.), PEI 25 kDa (PEI), PEIW, PEIL, PEIF, PEIY and PEIY with 20% tyrosine modification ($PEIY_{20}$). Upper panel: Luciferase activity shown as fold increase in RLU compared to untreated cells. Each bar represents a mean $\pm$ SD of three independent experiments. Lower panel: Corrected mRNA levels from transfections with ASO formulated in the same way, and bands follow the same order as in the upper panel.

nucleotides, even when using higher concentrations than those that were here applied.[33,36] A 5-fold increase in luciferase activity was the maximum achieved when using other polymers such as polyamidoamine (PAMAM),[37,38] whereas DLS liposomes induced a 45-fold increase in luciferase activity.[39]

Although PEI is known as a powerful polycation for plasmid DNA delivery, its polyplexes with short oligonucleotides were shown to be unstable, classifying it as a relatively poor carrier for this type of nucleic acids. It was recently found that providing PEI with mild hydrophobic properties would endow it with the ability to self-assemble, thereby stabilizing the particle formulation with siRNA.[16,22] Similar trends have been reported, and numerous studies have shown that modification of parental, highly cationic molecules by the addition of a hydrophobic moiety, or the attribution of self-assembly properties, improves their delivery.[40−45] Stability of those molecules has been studied using different methods and models.[16,46,47] By challenging our complexes with the competing anion heparin, we have verified that the incorporation of amino acids into PEI transformed it into

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significantly different polymers with varied cohesive properties. PEIW and PEIF showed very slow release rates, stably retaining 50% of the total ASO upon increased concentrations of heparin. This phenomenon can be explained by the assembly of ASO into extremely resistant shell-like particles, that hinders successful release. Although, under equal conditions of incubation time and temperature, PEIY showed a slow release at low concentrations of heparin, it allowed for more than 80% of the ASO to disassemble at the higher heparin concentrations. On the other hand, PEIL and unmodified PEI were characterized by a greater instability as indicated by the too fast ASO release rate. Indeed, stability plays an important role in any delivery system since the cargo must be protected from degradation but at the same time needs to be released to exert its effect. As a consequence, carriers that strongly bind to their cargos may hamper their activity by creating shell-like particles unable to dissociate.[48,49] This phenomenon might provide an explanation for the low activity of the complexes resulting from PEIW and PEIF. Equal reasoning, but the opposed extreme situation, applies to the ASO particles formed by PEI and PEIL.

The data obtained by the heparin assay was supported by the results from the dynamic light scattering studies, which showed that PEIY, PEIW and PEIF were capable of forming stable nanoparticles when combined with the ASO. The polyplex-formed particle populations were shown to be resistant to serum-induced aggregation, remaining stable over time and maintaining their integrity regarding homogeneity as well as average size. However, differences between the PEIY/ASO population and those of PEIW/ASO and PEIF/ASO were significant, with the later ones presenting a larger mean diameter. For activity purposes, exon-skipping ASOs must be delivered to the cell nucleus and, thereby, size can be a limiting factor. Similarly to what was found with the heparin competition assay, and contrary to Y, W and F-modified PEIs, the results of the DLS studies for the parental PEI as well as for PEIL suggested an immediate dissociation of the oligonucleotide complexes upon dilution in OptiMEM containing 10% FBS, most likely owing to ionic competition. Altogether, the discrepancies in size and stability between the PEI derivatives suggest an explanation for their difference in activity. Therefore, the specific amino acid modification must be taken into consideration and the way these polymers interact with the oligonucleotides has yet to be described. Several other factors can affect the activity of a delivery vector such as zeta potential, conformation of the complexes, binding affinity and receptor-mediated cell internalization.[28,46,50] However, in the present report we have only characterized the PEI derivatives regarding cytotoxic properties, particle size and stability while attempting to establish a correlation between these and the activity of their oligonucleotide complexes.

In contrast to what we found for ASO formulations, PEIW and PEIF were earlier shown to be relatively active in delivering siRNA.[22] In that study, PEIW became even more efficient when the tryptophan content was decreased to 20% ($PEIW_{20}$). However, the same reduction in the amino acid content did not improve the capacity of PEIW to deliver splice-correcting oligonucleotides. Indeed, the discrepancy of the various PEI polymers between siRNA and exon-skipping ASO delivery is clear and, in fact, not so surprising since the two gene silencing technologies rely on oligonucleotides of different chemistry as well as on delivery into different intracellular locations. While siRNA is frequently composed of unmodified bases, splice oligonucleotides constitute of 2′-*O*-methyl RNA bases with phosphorothioate backbone. It was previously shown that the effectiveness of many cationic lipids and peptides depends on the phosphodiester/phosphorothioate nature of the oligonucleotide cargo as well as on the chemistry of the bases.[51,52] In addition, as earlier mentioned, splice correction, occurring in the nucleus, may be more stringent to vectors than RNAi for which the target (the RISC) is located in the cytosol.[53]

The optimal conditions for formulation of PEIY polyplexes were found to be at a ratio 20:1 (+/−). Further increases in the PEIY concentration resulted in nonsignificant augmentation of splice correction at the mRNA level and no further enhancement of the protein activity. This phenomenon has been shown before, when PEIY was used for siRNA formulation[22] and was explained as the result of dilution of the ASO onto uninternalized PEIY complexes and/or a consequence of the saturation of cellular receptors by the polyplexes. A third explanation might be related to an increase in toxicity, since at ratio 30:1 the concentration of PEIY present is already half of the calculated IC_{50} (0.135 mM). However, at the charge ratio of 20:1, no significant effect on the cell proliferation was detected. Moreover, and interestingly, even at concentrations of the oligonucleotides as low as 0.025 μM, PEIY was able to induce approximately 50% splice correction on the mRNA level. This demonstrates the high efficiency of this newly developed vector and its

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potential, since under these conditions it causes no toxicity and allows the use of a minimal amount of oligonucleotides. Moreover, if higher concentrations of PEIY are required, possible cytotoxic effects may be decreased by adding PEG molecules, for instance. Inclusion of PEG to unmodified PEI and other polymer-based transfection reagents has shown improvement of their *in vivo* performance, also by reducing its toxicity.[20,54,55] When the amino acid content in PEIY was reduced from 30% down to 20%, the effect of the ASO delivered significantly decreased. This reduction in efficiency was observed in splice correction assays on both the protein and the mRNA levels. This phenomenon indicates that a certain hydrophobic/hydrophilic balance is required for keeping the effectiveness of the modified polymer.

Conclusion

Herein, we have evaluated several amino acid modified PEIs for the delivery of single-stranded oligonucleotides and identified tyrosine-modified PEI as a potent carrier for antisense 2′-*O*-methyl phosphorothiaote splice-correcting oligonucleotides. We have verified the relevance of hydrophobic contributions for the stability and efficiency of this type of polyplexes. When formulated in optimal conditions, at the ratio of 20:1, and in the presence of serum, polyplexes of PEIY/ASO presented a hydrodynamic mean of 133 nm and resulted in approximately 85% correction of the aberrant mRNA and an increase in protein activity of 450-fold. PEIY was even effective at the concentration of 0.025 μM of oligonucleotides, with no cytotoxic effect being detected. Importantly, we were able to show strong correlations between the size and stability of the ASO nanocomplexes and their activity. Furthermore, a good balance between extracellular stability and intracellular disassembly characterized PEIY, suggesting this polymer as a promising candidate for *in vivo* delivery and experiments are currently undertaken toward this direction.

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Supporting Information Available: Assessment of particle size by dynamic light scattering. This material is available free of charge via the Internet at http://pubs.acs.org.

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