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EFFICIENT DELIVERY OF INTACT PHOSPHODIESTER OLIGONUCLEOTIDES BY POLY- β -AMINO ESTERS

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Running head: PBAE's for delivering PO-ODNs

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ABSTRACT

Due to their great instability, phosphodiester antisense oligonucleotides (PO-ODNs) are rapidly degraded in the intracellular environment, which limits their biological activity. The release of PO-ODNs during a prolonged period of time could however greatly enhance their antisense effect by creating a pool of intact PO-ODNs at any time point. Poly- β -aminoesters are biodegradable cationic polymers which show potential for the controlled release of short DNA fragments like ODNs and small interfering RNA (siRNA). In this research we evaluated biodegradable poly- β -aminoesters as carriers for PO-ODNs and compared the antisense activity with nuclease stable phosphothioate (PS) ODNs. PBAE1 polymers were not able to generate an antisense effect with PO- or PS-ODNs, most likely due to their poor cellular uptake. When complexed to PBAE2 polymers at N/P ratio 10, both PO- and PS-ODNs downregulated the targeted protein expression with 70%. By confocal imaging we observed a high concentration of released PO-ODNs that formed nuclear bodies in the nucleoplasm. The ODNs in this nuclear bodies were still intact as could be demonstrated by Fluorescence Resonance Energy Transfer (FRET) and acceptor photobleaching. This was in clear contrast to PO-ODNs delivery by cationic liposomes where the ODNs that accumulated in the nucleus were degraded and nuclear bodies were not observed. We conclude that PBAE2 shows potential for the delivery of nuclease sensitive PO-ODNs. This occurs however not through a time controlled release profile, but rather due to the rapid delivery of a high concentration of intact PO-ODNs that form nuclear bodies in the nuclei of the cells. These nuclear bodies can most likely act as a depot of intact PO-ODNs, resulting in efficient antisense activity.

keywords: poly β aminoesters, Fluorescence Correlation Spectroscopy, Fluorescence Resonance Energy Transfer, oligonucleotides, nucleases, delivery

INTRODUCTION

Delivery of small DNA fragments such as antisense oligonucleotides (ODNs) and small interfering RNA (siRNA) remains an attractive research domain to achieve the therapeutic regulation of gene expression. Antisense oligonucleotides are short single stranded DNA strands which naturally consist of a phosphodiester (PO) backbone. Due to the high instability of this PO backbone, however, the routinely used ODNs consist of the more stable phosphothioate (PS) backbone (1;2). ODNs can act both in the cytoplasm and the nucleus by a sequence-specific recognition of the complementary mRNA. Through a variety of mechanisms, the complementary mRNA is then destroyed which results in the downregulation of the corresponding protein (3;4).

siRNAs are short double-stranded RNA fragments with a 2 nucleotides overhang at the 3' end. They are very potent molecules since they are recognised by intracellular RNA-induced silencing complexes (RISCs) which specifically and repeatedly destroy the targeted mRNA molecules. siRNA can be used in much lower concentrations and generally achieves a higher knockdown efficiency when compared to ODNs (5;6).

Both ODNs and siRNA need to reach the cytoplasm or nucleus of the cells before a biological effect can be obtained. As naked ODNs and siRNA are not efficient in crossing the cellular membrane, they are mostly complexed to carriers such as cationic liposomes or polymers (1). The positively charged complexes can relatively easily enter the cells by endocytosis but then still need to escape from the endosomal compartment and release the ODNs or siRNA in the intracellular environment. To allow us to explore the therapeutic potential of the simple mechanism of antisense therapy, the search for suited gene delivery carriers continues.

Previously we demonstrated that cationic liposomes are suitable for delivery of PS- but not PO-ODNs (7). We found that cationic liposomes deliver the ODNs they were carrying upon endosomal escape and that the released ODNs subsequently accumulated rapidly in the nuclei of the transfected cells. We showed experimentally that in the case of a PS backbone the delivered ODNs remained intact while PO-ODN became degraded by intracellular nucleases before a biological effect could be obtained. As the delivery of PO-ODNs remains attractive, we reasoned that due to their rapid degradation, successful delivery would only be possible with carriers that slowly release the ODNs during a prolonged period of time, thus creating a pool of intact PO-ODNs at any time during the delivery process.

In this research we evaluated the use of biodegradable poly- β -aminoesters as carriers for PO-ODNs. Poly- β -aminoesters are cationic polymers that spontaneously form nanocomplexes with the negatively charged DNA molecules and show broad potential for DNA delivery both for *in vitro* and *in vivo* applications (8;9). Apart from a positive charge, the presence of tertiary amines gives the polymers a certain buffering capacity, which is favourable to enhance endosomal escape by the so-called "proton sponge" effect (10). Also, the hydrolysable ester bonds make them interesting candidates for the controlled release of DNA due to polymer backbone degradation. Furthermore, biodegradable polymers are potentially more biocompatible for long-term or repeated applications. We previously demonstrated that poly- β -aminoesters are suitable for the long-term delivery of siRNA to hepatoma cells, probably because they may create a depot of complexed siRNA that is slowly being released in the cytoplasm of the cells (11). Therefore we wanted to evaluate if these carriers would be suitable to deliver intact PO-ODNs during a prolonged period of time, resulting in a certain extent of protein downregulation.

MATERIALS AND METHODS

Materials

For cytotoxicity and antisense activity measurements, anti-ICAM-1 20mer ODNs with a phosphodiester backbone (PO-ODN) or a phosphothioate backbone (PS-ODN) were used (Isis1939: 5' CCC-CCA-CCA-CTT-CCC-CTC-TC 3'). For the Fluorescence Correlation Spectroscopy (FCS) experiments and confocal imaging, the Isis1939 PS-ODNs were double-labeled with a rhodamine green fluorophore at the 3' end ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 532 \text{ nm}$) and a Cy5 fluorophore at the 5' end

($\lambda_{\text{ex}} = 647 \text{ nm}$, $\lambda_{\text{em}} = 670 \text{ nm}$). As a PO-ODN, a double-labeled 40 mer phosphodiester ODN (5' GCC-GTC-TCT-GAC-TGC-TGA-TGA-CTA-CTA-TCG-TAT-AGT-GCG-G 3'; 13388,1 g/mol) was used, since we demonstrated in previous research that this 40 mer PO-ODN has a higher FRET efficiency when compared to the double-labeled Isis1939 PO-ODNs (12). It should be noted that the difference in length does not interfere with the complexation and dissociation profile of the PO-ODNs, nor does it influence the intracellular distribution upon microinjection or transfection experiments. All ODNs (labeled and non-labeled) were purchased from Eurogentec (Seraing, Belgium) and were purified by polyacrylamide gel electrophoresis (PAGE) by the supplier. In the intact double-labeled ODNs used in this study Fluorescence Resonance Energy Transfer (FRET) occurs between the rhodamine green fluorophore on the 3' end and the Cy5 fluorophore on the 5' end (12).

The polymers used in the experiments are biodegradable poly(β -amino esters), namely PBAE1 and PBAE2 (the molecular weight of the monomers is respectively 408 g/mol and 436 g/mol) (Figure 1). PBAE1 and PBAE2 had an average molecular weight of respectively 18 kDa and 22 kDa and were synthesized as described elsewhere (11). As these PBAE's were not soluble at a neutral or basic pH, they were dissolved in 0.1 M acetate buffer (pH 5.4) and were kept at -20°C to avoid hydrolysis of the ester functions of the polymers after production.

DNase I (Pulmozyme®, 1 unit/ μl) was kindly provided by NV Roche, Brussels, Belgium. Dextran sulfate (DS) was purchased from Sigma (St Louis, USA). The molar mass and sulfate content, as provided by the supplier, equaled respectively 500 kDa and 2.3 sulfate groups per glucosyl residue. A stock solution of 10 $\mu\text{g}/\mu\text{l}$ was prepared in 20 mM Hepes buffer. Lipofectamine™ 2000 was purchased from Invitrogen (Merelbeke, Belgium).

Human lung carcinoma cells (A549 cells, ATCC number: CCL-185) (DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM) without phenol red (Gibco, Merelbeke, Belgium) containing 2 mM glutamine, 10% heat deactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 .

Figure 1

Schematic representation of PBAE1 (A) and PBAE2 (B) as used in this study. The molecular weight of PBAE1 and PBAE2 monomers is respectively 408 g/mol and 436 g/mol. The molecular weight of one nitrogen containing monomer was calculated as half of the molecular weight due to symmetrical nature of polymers (A) and (B).

Preparation of PBAE1/ODN and PBAE2/ODN polyplexes

The N/P ratio of the polyplexes is defined as the molar ratio of the total number of nitrogen atoms of the PBAE polymers to the number of DNA phosphates. Polyplexes with different N/P ratios were prepared by adding a cationic polymer solution to an equal volume of an ODN solution, followed by vortexing the dispersion for 10 seconds. Every polymer and ODN solution was diluted in 0.1 M acetate buffer (pH 5.4) prior to mixing, to assure that the polymer was soluble and able to interact with the ODNs. The polyplexes were allowed to equilibrate at room temperature for 30 minutes prior to use. Then, for gel electrophoresis, FCS measurements and ODNs degradation experiments, the complexes were further diluted in the so called “degradation buffer” (2 mM magnesium acetate, 110 mM potassium acetate and 20 mM Hepes, pH 7.4). In this buffer it is sure that the enzyme DNase I is active. For cytotoxicity, uptake, antisense activity and acceptor photobleaching experiments, the complexes were diluted in Opti-MEM® instead of degradation buffer before adding the complexes to the cells. The hydrodynamic size and zeta potential of the

polyplexes was checked by respectively dynamic light scattering and surface potential measurements (NanoZS, Malvern, Worcestershire, UK), as previously described (13).

Gel electrophoresis measurements on the polyplex dispersions and ODNs degradation

To study the complexation between ODNs and respectively PBAE1 and PBAE2 by agarose gel electrophoresis, 10 μ l of the ODN solution (60 μ g/ml) was mixed with 10 μ l of a PBAE1 respectively PBAE2 solution (the concentration being dependent on the desired N/P ratio). The polyplexes were allowed to equilibrate for 30 minutes before further use. To study the displacement of the ODNs from the polyplexes an increasing amount of dextran sulfate was added to the dispersions. All samples were diluted with degradation buffer to a total volume of 30 μ l. Also, 5 μ l was taken from each sample and diluted with degradation buffer to a total volume of 50 μ l to perform FCS measurements. Before loading the remaining samples on the 1% agarose gel, 5 μ l of 50% sucrose was added.

The degradation of the 40 mer ODNs, upon exposure of the polyplexes to DNase I, was studied by polyacrylamide gel electrophoresis (PAGE) as follows. 2 μ l of the ODN solution (100 μ g/ml) was mixed with 2 μ l of the PBAE1 (1219 μ g/ml) respectively PBAE2 (1302 μ g/ml) solution, resulting in polyplexes with N/P ratio 20. After equilibration, the samples were diluted with degradation buffer to a total volume of 12 μ l. Then, the samples were incubated, respectively with or without 1 unit of DNase I, during 24 hours. After the desired incubation time, 5 μ l 10x EDTA-enriched TBE buffer was added (10.8 g/l tris base, 5.5 g/l boric acid and 3.7 g/l EDTA) to inhibit DNase I. Subsequently, 5 μ l of dextran sulfate (10 μ g/ μ l) was added to release the ODNs from the polyplexes before loading the samples on the polyacrylamide gel. Also, from these dispersions 5 μ l was removed and diluted with degradation buffer to a total volume of 50 μ l to perform FCS measurements. Before loading the remaining samples on the polyacrylamide gel, 5 μ l of 50% sucrose was added.

FCS measurements on the polyplex dispersions and ODNs degradation

Dual-color FCS measurements were performed on polyplexes composed of non-labeled cationic polymers (PBAE1 or PBAE2) and double-labeled 40 mer PO-ODNs. A dual-color FCS-setup installed on a MRC1024 Bio-Rad confocal laser scanning microscope was used. An inverted microscope (Eclipse TE300, Nikon, Japan) was used, which was equipped with a water immersion objective lens (Plan Apo 60X, NA 1.2, collar rim correction, Nikon, Japan). To verify whether the excitation volumes and the detection volumes optimally overlapped, the system was calibrated as described by Schwille et al. (14). The laser beam was focused at about 50 μ m above the bottom of the glass-bottom 96-well plate (Grainer Bio-one, Frickenhausen, Germany), which contained the samples. The 488 nm and the 647 nm laser beams of a krypton-argon laser (Bio-Rad, Cheshire, UK) were used and the green and red fluorescence intensity fluctuations were recorded on a digital ALV 5000/E correlator while exciting the polyplexes at 488 or 647 nm during 3 intervals of 50 seconds. To evaluate the integrity of the ODNs, the ratio of the red to the green fluorescence (R/G ratio) was calculated after release of the ODNs from the polyplexes by dextran sulfate upon excitation with 488 nm. This R/G ratio is a measure for the amount of intact ODNs, as previously described (12). From the R/G ratio, the amount of degraded ODNs can be calculated using the equation $y = 1.0437e^{-0.035x}$ in which y is the normalized R/G ratio and x is the percentage of degraded ODNs. This equation was determined by measuring the R/G ratio of mixtures of intact and degraded ODNs in solutions containing an increasing percentage of degraded ODNs (from 0, 20, 40, 60, 80 and 100%) and plotting the normalized R/G ratio in function of the percentage of degraded ODNs (15).

To follow the degradation of the complexed ODNs in function of the time (Figure 4), polyplexes were prepared by mixing 100 μ l 2 μ M ODNs with 100 μ l polymer solution (respectively 163.2 or 326.4 μ g/ml PBAE1 and 174.4 or 348.8 μ g/ml PBAE2 to obtain N/P ratio 10 or 20). After 30 minutes equilibration, the polyplex dispersions were diluted with degradation buffer to a total volume of 1000 μ l and subsequently divided into two aliquots of 500 μ l. To one 500 μ L aliquot 2 μ l (2 units) DNase I was added. The two aliquots were further divided into eppendorfs containing

each 50 µl polyplex dispersion and incubated at 37 °C. After the desired incubation time, 50 µl 2X EDTA-enriched TBE buffer was added to the eppendorfs to inhibit the DNase I. On these samples, FCS measurements were performed after addition of 10 µl dextran sulfate (10 µg/µl) to release the ODNs from respectively the PBAE1 and PBAE2 polyplexes.

Cytotoxicity of PO- and PS-ODNs containing PBAE polyplexes

The cell viability in the presence of free PBAE polymers and PBAE/ODN complexes was determined using the EZ4U assay (Biomedica, Vienna, Austria). 5×10^4 cells were seeded per well of a 96-well plate and allowed to adhere overnight. After 24 hrs, cells were washed with PBS and incubated with 100 µl free ODN, free polymer or PBAE/ODN complexes with N/P ratio 10 and 20 (at 0.7 µg ODN/well) in Opti-MEM[®]. After 4 hrs, the cells were washed with PBS and incubated with culture medium for 24 hours. Then, cells were washed again with PBS and incubated with a mixture of 20 µl EZ4U substrate and 180 µl culture medium. After 4 hrs incubation at 37°C reduced formazan was measured on a plate reader at 450 nm and 620 nm (Wallac Victor2 multilabel reader, Perkin-Elmer Life Sciences, Boston, USA).

Antisense activity of PO- and PS-ODNs containing PBAE/ODN complexes

The antisense activity of non-labeled phosphodiester (PO) and phosphothioate (PS) anti-ICAM-1 ODNs was determined by detecting the ICAM-1 expression on the surface of A549 cells using ELISA. Mycoplasma free A549 cells were plated onto 96-well microtiter plates at 5×10^4 cells/well. At 90% confluency, the cells were washed three times with phosphate buffered saline (PBS) and PBAE/ODNs complexes with N/P ratio 10 or 20 were added to the cells (0.7 µg ODN/well) in Opti-MEM[®]. Also naked ODNs and ODNs complexed with lipofectamine[™] 2000 (Invitrogen, Merelbeke, Belgium) were included. After 4 hours of incubation, the free or complexed ODNs were removed and cells were washed with PBS and incubated with 100 µl culture medium supplemented with 10 ng/mL TNF-α to induce overexpression of ICAM-1. After 18 hours of incubation, the ICAM-1 expression was determined as described before (7). To determine the basal ICAM-1 expression, the cells were incubated for 18 hours with 100 µL culture medium without TNF-α.

Uptake of PBAE/ODN complexes and acceptor photobleaching

Confocal imaging and acceptor photobleaching experiments were performed on a LSM510 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany). Detection of the emission light was obtained with photonmultipliers and confocal detection was ensured by excluding out-of-plane fluorescence with pinholes in front of the detectors (70 µm or 90 µm for respectively the green or the red detector). For acceptor photobleaching experiments, a “pre-bleaching” image was taken with laser excitation set to 488 nm and 633 nm. Then, the Cy5 acceptor was locally bleached at 10x zoom by increasing the 633 nm laser excitation to 100%. When bleaching was completed (typically some 30 seconds) a “post-bleaching” image was taken at the original zoom and with laser excitation set to 488 nm and 633 nm as in the “pre-bleaching” image. From these images, the FRET efficiency in the bleached region was calculated from the increase in donor emission after acceptor photobleaching as follows: $\% \text{ FRET} = (F_{\text{donor post}} - F_{\text{donor pre}}) / F_{\text{donor post}}$ with F being the average fluorescence intensity measured in the “post-bleaching” and “pre-bleaching” donor image. FRET efficiencies were calculated in Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2009.)

The uptake of PBAE/ODN complexes was followed by flow cytometry. Briefly, polyplexes composed of non-labeled cationic polymers (PBAE1 or PBAE2) and rhodamine green-labeled anti-ICAM PO- or PS-ODNs were incubated with A549 cells at 90% confluency during 0.5, 1, 2, 3 or 4 hours in Opti-MEM[®] at 37°C and 5% CO₂. After the appropriate time, extracellular fluorescence was quenched with trypan blue during 10 minutes, the cells were washed 3 times with PBS and incubated with trypsin-EDTA to detach the cells from the wells. After 5 minutes, 1 ml culture medium was added and the cell suspension was centrifuged during 6 minutes at 1400 rpm. The cell

pellet was then resuspended in 300 μ l flow buffer (1% BSA in PBS + 1 U DNaseI) and the amount of (green) cells that had taken up the polyplexes was determined by a BD FACSCalibur™ Flow Cytometer (BD Biosciences, Erembodegem, Belgium). As a controle, non-transfected cells, or cells incubated on ice with the polyplexes were used.

RESULTS AND DISCUSSION

Formation of PBAE1/ODN and PBAE2/ODN complexes

The association of ODNs to PBAE1 and PBAE2, in function of the N/P ratio, was studied by agarose gel electrophoresis. As Figure 2A shows, until N/P ratio 1, free (uncomplexed) ODNs migrate in the agarose gel indicating that the amount of cationic polymer is not sufficient to bind all the (negatively charged) ODNs. At N/P ratio 2.5 ODNs do no longer migrate in the gel, indicating that all the ODNs are complexed to the positively charged PBAE polymers.

As we demonstrated before, FCS is an easy tool to determine the association and dissociation of ODNs to cationic carriers (13;16-18). Briefly, a FCS setup monitors the fluctuations in fluorescence intensity in the confocal volume of a microscope. These fluctuations are coming from fluorescently labeled molecules (in this study ODNs) which diffuse in and out the confocal volume. In solutions containing only free ODNs, the fluorescence in the confocal volume fluctuates slightly around a mean value (see left panel in Figure 2C). When, however, the ODNs become complexed, highly intense fluorescence peaks arise in the fluctuations profile (see middle panel of Figure 2C). Indeed, when a complex containing multiple ODNs passes the detection volume of the FCS instrument, many ODNs are detected at once, resulting in a highly intense fluorescence peak. The fluorescence fluctuations which are seen between such highly intense peaks (so named 'baseline fluorescence') are due to the ODNs which remain free in the solution. One can thus calculate the percentage of free ODNs from this baseline fluorescence (19).

The complexation between ODNs and PBAE polymers could be easily monitored by FCS measurements (Figure 2C). Before complexation, upon excitation with 488 nm free (double labeled) ODNs displayed fluorescence fluctuations around a mean intensity value of 13 ± 1 kHz and 123 ± 2 kHz for respectively the green and the red channel (Figure 2C, left panel), resulting in a red to green (R/G) ratio of 9.5 ± 1 . FRET could thus clearly be detected in the intact ODNs. Upon complexation however, the fluorescence baseline dropped and fluorescence peaks arised in the fluctuation profile (Figure 2C, middle panel), indicating that multiple ODNs were indeed bound to the PBAE polymers in a multicomponent complex. It is important to note that in the complexes, the R/G ratio does not represent the integrity of the ODNs anymore, as the position of the donor and acceptor fluorophores is not only determined by the integrity of the ODN backbone, but also by the relative positions of the ODNs in the complexes.

Figure 2

(A) Gel electrophoresis on free PO-ODNs and PBAE1/PO-ODN complexes at different N/P ratios as depicted above each lane. (B) Gel electrophoresis on PBAE1/PO-ODN complexes (N/P ratio 10) dissociated by adding increasing amounts of dextran sulfate as depicted above each lane. The amount of ODNs in each lane was 300 ng. (C-D) FCS measurements on a fraction of the samples loaded on respectively gel A and gel B as described in the materials and methods section. Laser excitation was set to 488 nm and the fluorescence intensities were recorded by the green (green) or red (red) detector. In the right graph, the blue data points represent the R/G ratio. No differences in the association or dissociation of PBAE2/ODNs complexes was observed when compared to the gels for PBAE1/ODN complexes depicted in respectively (A) and (B) and the FCS measurements depicted in (D).

When negatively charged dextran sulfate is added to the polyplex dispersions, it is expected that it will compete with the ODNs for binding to the PBAE1 and PBAE2 polymers, thereby displacing the ODNs from the polyplexes. This was indeed observed by gel electrophoresis. Release of ODNs from both PBAE1 and PBAE2 starts from 3 μg dextran sulfate onwards (Figure 2B). Also FCS measurements could be used to follow the dissociation of the ODNs from their carriers. When more ODNs were released from the carrier in the surrounding solutions, the baseline fluorescence gradually increased while highly intense peaks became less frequently observed in the fluorescence fluctuation profile. Eventually, the baseline fluorescence recovered until the level of free ODNs and fluorescence peaks did not longer occur. By FCS we observed the release of the ODNs from the complexes starting from 1 μg dextran sulfate (Figure 2D). Importantly, not only the fluorescence baseline, but also the R/G ratio gradually recovered until the value as measured for free ODNs, as expected (Figure 2D). It should be noted that spontaneous ODN release from the polyplexes can occur due to backbone degradation of the biodegradable PBAE1 and PBAE2 polymers. We followed this spontaneous release at pH7 and 37°C by measuring the fluorescence intensities of PBAE1/ODN or PBAE2/ODN complex solutions at NP ratio 10 in function of time with the FCS instrument. Spontaneous release of the ODNs started after 2 days of incubation of the polyplexes and was completed after an incubation period of 4 (PBAE1) or 6 (PBAE2) days (data not shown).

Hydrodynamic size and zeta potential of PBAE1/ODN and PBAE2/ODN polyplexes

Table 1 shows the particle size and zeta potential of different kind of PBAE/ODN polyplexes. Although gel electrophoresis demonstrated that complex formation already starts from N/P 2.5, size measurements pointed out that at this N/P ratio very large complexes were formed. As the zeta potential (which also determines the repulsion between the polyplexes) is lower when compared to higher N/P ratios, this is probably due to aggregation of different polyplexes. At higher N/P ratios, smaller complexes were formed, indicating better compaction of the ODNs and less aggregation of the formed polyplexes. Apparently a zeta potential of about 25 mV causes sufficient repulsion between the polyplexes to prevent aggregation.

Table 1: The particle size and zeta potential of the PBAE/PS-ODN complexes prepared at different N/P ratios (Mean $\pm$ standard deviation of three measurements). Similar results were obtained with PBAE/PO-ODN complexes.

	N/P 2.5	N/P 10	N/P 20	N/P 50
Particle size (nm)				
PBAE1/ODN	1240 $\pm$ 105	156 $\pm$ 5	149 $\pm$ 7	126 $\pm$ 3
PBAE2/ODN	1235 $\pm$ 182	245 $\pm$ 5	147 $\pm$ 1	140 $\pm$ 5
Zetapotential (mV)				
PBAE1/ODN	15 $\pm$ 1	25 $\pm$ 1	20 $\pm$ 1	22 $\pm$ 2
PBAE2/ODN	16 $\pm$ 1	26 $\pm$ 1	25 $\pm$ 1	20 $\pm$ 1

Protection of PO-ODNs against enzymatic degradation by PBAE1 and PBAE2

The DNase induced degradation of PO-ODNs complexed to PBAE1 and PBAE2 polymers was initially studied by PAGE gel electrophoresis (Figure 3). Therefore, the complexes were dispersed in buffer containing 1 unit of DNase I for 24 hours and then dissociated by the addition of dextran sulfate, after the inhibition of the DNase I enzyme. Upon degradation of naked PO-ODNs, the smaller degradation products run further in the gel when compared to intact PO-ODNs (Figure 3, lane 2). When complexed to PBAE1, only 27% of the PO-ODNs survived a 24 hours incubation in a DNase solution (Figure 3, lane 4), while when complexed to PBAE2 83% of the PO-ODNs remained intact after the 24 hours incubation (Figure 3, lane 6). This clearly points out that PBAE2 offers a better protection to the complexed PO-ODNs, when compared to PBAE1.

Figure 3

Gel electrophoresis on free PO-ODNs, PBAE1/PO-ODN and PBAE2/PO-ODN dispersions. Lane 1-2: free PO-ODNs respectively without and with 1 unit DNase I, lane 3-4: PBAE1/PO-ODN polyplexes respectively without and with 1 unit DNase I and lane 5-6: PBAE2/PO-ODN polyplexes respectively without and with 1 unit DNase I. The N/P ratio of the polyplexes was 20. The samples were incubated at 37°C during 24 hours. Before loading the samples on the gel, the DNase I activity was inhibited and the PO-ODNs were released from the polyplexes by adding dextran sulfate. The % of intact PO-ODNs as calculated from the R/G ratio as determined by FCS on a fraction of the samples is also depicted. Each lane contained 300 ng PO-ODNs.

We then studied the protection against enzymatic degradation with FCS in more detail for PBAE/PO-ODN complexes with N/P ratio 10 and 20 (Figure 4). Therefore we measured the R/G ratio of the PO-ODNs (upon excitation with 488 nm) released by dextran sulfate from polyplexes after being incubated with DNase I for a certain period of time. From the R/G ratio, the amount of degraded PO-ODNs was calculated as described in the materials and methods section. When no DNase I was added, degradation of the complexed PO-ODNs was not observed. Indeed, $98 \pm 2\%$ of the PO-ODNs remained intact during the 6 days incubation period at 37 °C (Figure 4, squares). When the complexes were incubated with DNase I, degradation of the PO-ODNs was observed. In PBAE2 complexes the PO-ODNs seemed better protected when compared to PBAE1, thus suggesting that PBAE2 complexes are better suited to deliver intact PO-ODNs during a long period of time. Also, it should be noted that PO-ODNs seem better protected in complexes with a higher N/P ratio, which most likely results from a better compaction of the PO-ODNs and a subsequent shielding of the PO-ODNs from the DNase I enzyme in the surrounding environment.

Figure 4

Percentage of PO-ODNs remaining intact after incubating the PBAE1/PO-ODN and PBAE2/PO-ODN polyplexes with DNase I, as measured by FCS. The percentage of intact PO-ODNs was calculated from the R/G ratio, after inhibition of the DNase I and subsequent release of the PO-ODNs from the polyplexes using dextran sulfate. Laser excitation was 488 nm. Polyplexes incubated without DNase I (average $\pm$ standard deviation of N/P ratio 10 and 20). PBAE1/PO-ODN polyplexes with N/P ratio 10 (●) and 20 (▲) incubated with 1 unit of DNase I and PBAE2/PO-ODN polyplexes with N/P ratio 10 (▼) and 20 (◆) incubated with 1 unit of DNase I. Values represent the average $\pm$ standard deviation of two samples per time point. When no error bars appear, they were smaller than the symbol.

Cytotoxicity, biological activity and cellular uptake of PO- and PS-ODNs delivered by PBAE1 and PBAE2 polymers

The cytotoxicity of free ODNs, free PBAE polymers and PBAE/ODN complexes was determined by the EZ4U assay, which relies on the reduction of tetrazolium salt to colored formazan by dehydrogenases present in the mitochondria of living cells (Figure 5). There was no cytotoxicity when the cells were incubated with DMEM or free ODNs. Also, the cytotoxicity was acceptable when lipofectamine/ODN complexes or free PBAE1 were used. Free PBAE2 seemed more toxic than free PBAE1. Less cell death was observed when the polymers were complexed to PO- or PS-ODNs. When 30% cell death can be tolerated, PBAE1/ODN complexes (at N/P ratio 10 and 20) and PBAE2/ODN complexes at N/P ratio 10 are acceptable. The toxicity of PBAE2/ODN complexes at N/P ratio 20 seemed too severe. It should be noted that no significant differences could be observed between the cytotoxicity of PBAE/ODN complexes containing respectively PO- or PS-ODNs.

Figure 5

Cytotoxicity, as measured by the EZ4U test, of the free PO- and PS-ODNs, free PBAE1 and PBAE2 polymers (at a concentration needed to prepare complexes with N/P ratio 10 or 20) and different PBAE/ODN complexes at N/P ratio 10 or 20. A line was drawn at the level of 50% cytotoxicity. Values represent the average $\pm$ standard deviation of three samples per situation. No difference was observed between complexes containing PO- or PS-ODNs.

Next we determined the biological activity of free ODNs and PBAE/ODN complexes by measuring the downregulation of ICAM-1 expression at the surface of A549 cells (Figure 6). As Figure 5 pointed out that complexes at N/P ratio 20 are too toxic, only complexes at N/P ratio 10 were used. Lipofectamine/ODN complexes were included as a control in the transfection experiments as we know from previous experiments that lipid based carriers do transfect when PS-ODNs are delivered, but not when PO-ODNs are used (7). As Figure 6 shows, PO-ODNs delivered by lipofectamine indeed failed in the down regulation of the ICAM-1 expression, while PS-ODNs delivered by lipofectamine reached about 50% of downregulation, in agreement with our previous experiments (7). With PBAE1/ODN complexes at N/P ratio 10, the downregulation of ICAM-1 was 20% when PS-ODNs were used while with PO-ODNs there was no downregulation. When PBAE2/ODN complexes were used at N/P ratio 10, both PO-ODNs and PS-ODNs downregulated ICAM-1 expression with respectively 70% and 60%. The better protection of PO-ODNs in PBAE2 polyplexes, when compared to PBAE1 (Figure 4), may explain why PO-ODNs downregulate

ICAM-1 only when delivered by PBAE2 polymers. This can however not explain the differences observed for PS-ODNs delivered by respectively PBAE1 and PBAE2. Therefore, the uptake of PBAE/ODN polyplexes was followed by flow cytometry in function of time (Figure 7). As a control, cells with polyplexes were also incubated on ice, to prevent cellular uptake. As expected, free ODNs were not taken up by the cells (data not shown). The uptake of polyplexes composed of PBAE1 was however also limited: only about 30% of the cells took up the polyplexes after 2 hours of incubation, compared to about 90% of the cells when polyplexes prepared with PBAE2 were used. Both for PBAE1 and PBAE2 this amount did not increase significantly with longer incubation times, indicating that the cellular uptake was ‘complete’ within the first 2 hours of incubation. It should be noted that the intracellular uptake of ODNs delivered by Lipofectamine™ 2000 was comparable with that of PBAE2. When the cells were incubated with the polyplexes on ice, the uptake remained below 3.4%, indicating that the endocytosis pathways were indeed inhibited in this situation. Since cellular uptake is a prerequisite for biological activity, the limited uptake of the PBAE1 polyplexes most likely explains why they were not efficient in generating an antisense effect.

Figure 6

Biological activity of PO- and PS-ODNs delivered by PBAE1 and PBAE2 polyplexes at N/P ratio 10 measured by means of ICAM-1 downregulation. Values represent the average $\pm$ standard deviation of three samples per situation. Free PO- and PS-ODNs and PO- and PS-ODNs delivered by the commercially available Lipofectamine™ 2000 (Lip PO and Lip PS) were also included.

Figure 7

Uptake of PBAE/ODN complexes in A549 cells in function of time as measured by flow cytometry. As a control, complexes incubated on ice during 2 hours were also included. Complexes were prepared with PBAE1 (gray bars) or PBAE2 (black bars) at N/P ratio 10. Values represent the average $\pm$ standard deviation of three independent measurement. No difference was observed between the uptake of PBAE complexes containing PO- or PS-ODNs. The uptake of free ODNs remained below 0.5 % (data not shown). As a comparison, uptake of Lipofectamine™ 2000 delivered ODNs after 4 hours of incubation is also shown (white bar).

FRET-imaging to determine the integrity of PO- and PS-ODNs

To identify whether the intracellularly delivered ODNs are intact or degraded, acceptor photobleaching was used. In the intact ODNs used in this study, FRET occurs between the rhodamine green (donor fluorophore) and the Cy5 (acceptor fluorophore). This leads to a decrease in intensity from the donor fluorescence, which relates to the amount of energy transfer to the acceptor molecules. This decrease in intensity by FRET can relatively easily be measured by specifically bleaching the acceptor. Indeed, the photobleached acceptor molecules will no longer ‘accept’ the energy transfer from the donor molecules and the donor fluorescence will increase. From this increase, the amount of FRET can be calculated as mentioned in the materials and methods section.

To demonstrate the presence of FRET in intact ODNs, acceptor photobleaching was performed on intact PS-ODNs and intact PO-ODNs. Also, as a negative control degraded PO-ODNs (in which FRET cannot occur) were used. The panels in Figure 8 show respectively the acceptor and donor fluorescence before acceptor photobleaching (images A and B). Then, we

specifically destroyed the acceptor fluorophores in a certain part of the image (black squares in the C-images of Figure 8). For the intact PS- and intact PO-ODNs we can clearly see an increase in donor fluorescence (D images) corresponding to the region where the acceptor molecules were destroyed, what indicates that FRET indeed occurs in these intact ODNs. For degraded PO-ODNs, however, there is no difference in fluorescence intensity inside or outside the photobleached region, indicating that the donor fluorescence did not increase after acceptor photobleaching. Indeed, in the degraded ODNs the donor and acceptor molecules are no longer in close proximity from each other, which explains why FRET does not occur. From the increase in donor fluorescence FRET efficiencies could be calculated: $50 \pm 2\%$ for the intact PS-ODNs, $67 \pm 1\%$ for the intact PO-ODNs and $0.5 \pm 0.1\%$ for the degraded PO-ODNs.

Figure 8

Acceptor photobleaching in a dried solution of intact PS-ODNs (left), intact PO-ODNs (middle) and degraded PO-ODNs (right). The acceptor (Cy5) fluorescence ($\lambda_{\text{ex}} = 633 \text{ nm}$) and donor (rhodamine green) fluorescence ($\lambda_{\text{ex}} = 488 \text{ nm}$) are shown before (pre-bleach, A and B) and after (post-bleach, C and D) acceptor photobleaching. Detector settings and laser excitation during taking of the pre-bleach and post-bleach confocal images were kept constant. FRET efficiency was calculated from the donor increase in the bleached region as mentioned in the materials and methods section.

Intracellular distribution and integrity of PO- and PS-ODNs delivered by PBAE1 and PBAE2 polymers

In a next experiment we determined the integrity of the PO- and PS-ODNs delivered in the A549 cells by acceptor photobleaching. Figure 9 shows the intracellular distribution of **PO**-ODNs delivered by PBAE1 and PBAE2 polymers at N/P ratio 10. We can clearly see the presence of ODNs in the nucleus of the cells, indicating that both polymers rapidly released a fraction of their ODNs in the cytoplasm of the cells, followed by nuclear accumulation. It is indeed known that, once released from the carrier, free ODNs rapidly accumulate into the nucleus of the cells (20;21). The presence of nuclear bodies in the nuclei of the cells (being the highly intense fluorescent spots) indicates that a *high* concentration of PO-ODNs is delivered intracellularly. Nuclear bodies are indeed only formed at higher concentration of ODNs and are considered to be a means to sequester an excess of ODNs in the cells (22;23). Although the information on nuclear bodies in literature is mainly gathered for PS-ODNs, these data clearly show that also PO-ODNs can form nuclear bodies when they are introduced into the cells. When we performed acceptor photobleaching on the PO-ODNs in these nuclear bodies, we found that at least a fraction of the delivered ODNs were still intact: the donor fluorescence increased about 23% for PBAE1 and about 30 to 49% for PBAE2. This suggests that a larger fraction of the ODNs remains intact when delivered by PBAE2, when compared to PBAE1. Figure 4 indeed demonstrated that the PBAE2 polymers offer a better protection to complexed PO-ODNs when compared to PBAE1. The fact that intact PO-ODNs were found in the nuclei of the cells transfected with PBAE2 is in line with the antisense effect observed for the PBAE2/PO-ODN complexes.

Figure 9

Acceptor photobleaching of PBAE1 (left) and PBAE2 (right) complexes containing PO-ODNs at N/P ratio 10, 4 hours after applying the complexes to the cells. The acceptor (Cy5) fluorescence ($\lambda_{\text{ex}} = 633 \text{ nm}$) and donor (rhodamine green) fluorescence ($\lambda_{\text{ex}} = 488 \text{ nm}$) are shown before (pre-bleach, A and B) and after (post-bleach, C and D) acceptor photobleaching. Detector settings and laser excitation during taking of the pre-bleach and post-bleach confocal images were kept constant. The square indicates the photobleached region. The corresponding transmission image is also shown (upper panel: PBAE1/PO-ODN 10 and lower panel: PBAE2/PO-ODN 10), in which a white line is drawn around the nuclei.

Figure 10 shows the intracellular distribution of *PS*-ODNs delivered by PBAE1 and PBAE2 at N/P ratio 10. In these cells, the ODNs were not found in the nuclei, but rather seemed to remain into dotted structures in the cytoplasm, which are most likely the complexes, since free *PS*-ODNs are known to rapidly accumulate in the nucleus. This indicates that the PBAE polymers deliver *PS*-ODNs in a different way than PO-ODNs, with the PO-ODNs being more easily released. It has been reported that other polymers like poly-ethylene-imine (PEI) also formed stronger complexes with *PS*-ODNs when compared to PO-ODNs, which could explain the difference in the release profile (24). From time to time, *PS*-ODNs could be found in the nuclei of the cells, although not as frequently as the PO-ODNs (data not shown). Acceptor photobleaching pointed out that the *PS*-ODNs in the complexes were all intact: the FRET efficiency was even higher than for intact *PS*-ODNs in solution (e.g. between 53% and 76% in the complexes, compared to 50% in solution). Probably the compaction of *PS*-ODNs in the complexes brings the donor and acceptor fluorophores even closer together (when compared to the situation in free *PS*-ODNs), resulting in a higher energy transfer and thus a more pronounced increase in donor fluorescence upon acceptor photobleaching.

Figure 10

Acceptor photobleaching of PBAE1 (left) and PBAE2 (right) complexes containing PS-ODNs at N/P ratio 10, 4 hours after applying the complexes to the cells. The acceptor (Cy5) fluorescence ($\lambda_{\text{ex}} = 633 \text{ nm}$) and donor (rhodamine green) fluorescence ($\lambda_{\text{ex}} = 488 \text{ nm}$) are shown before (pre-bleach, A and B) and after (post-bleach, C and D) acceptor photobleaching. Detector settings and laser excitation during taking of the pre-bleach and post-bleach confocal images were kept constant. The square indicates the photobleached region. The corresponding transmission image is also shown (upper panel: PBAE1/PS-ODN 10 and lower panel: PBAE2/PS-ODN 10), in which a white line is drawn around the nuclei.

In previous research we showed that lipid based carriers like DOTAP/DOPE liposomes rapidly deliver the ODNs they are carrying, followed by their accumulation in the nucleus (7). However, with DOTAP/DOPE liposomes nuclear bodies were only observed with PS-ODNs and not with PO-ODNs. Also, the PO-ODNs that arrived in the nuclei after release from the DOTAP/DOPE liposomes were all degraded, as could be demonstrated by Fluorescence Correlation Spectroscopy. In the current study, as a lipid based carrier, we used the commercially available LipofectamineTM 2000 instead of the DOTAP/DOPE liposomes. Therefore, we checked the intracellular distribution and integrity of PO- and PS-ODNs delivered by LipofectamineTM 2000, to see if our previous observations on the ODN delivery by DOTAP/DOPE liposomes also holds for the commercially available lipid based carriers. Figure 11 shows the “pre-bleaching” and “post-bleaching” confocal images of cells transfected with LipofectamineTM 2000 containing PO- or PS-ODNs. Again the ODNs delivered by the lipid base carrier rapidly accumulate in the nuclei of the transfected cells. Also, it can be seen that PO-ODNs do not form nuclear bodies (in clear contrast to PO-ODNs delivered by PBAE polyplexes), while PS-ODNs do. When performing acceptor photobleaching in these cells, it could be confirmed that the delivered PO-ODNs were degraded (e.g. only 3% increase in donor fluorescence was seen, which was comparable with the ‘background’ donor increase as observed in the non-photobleached region). With PS-ODNs, nuclear bodies can indeed be found in some of the cells. Also, acceptor photobleaching clearly demonstrates the presence of intact PS-ODNs in the nuclei of the cells: in the photobleached region, the donor fluorescence increases about 52%, while only 5% increase was observed in the top left nucleus where no acceptor photobleaching was performed. It should be noted that the nucleus just above the acceptor photobleached region was also photobleached significantly, explaining why also here a donor increase of 46% could be measured. These data clearly show that the intracellular distribution and integrity of PO- and PS-ODNs does not significantly differ with the commercially available LipofectamineTM 2000 and the DOTAP/DOPE liposomes we used in our previous studies.

Figure 11

Acceptor photobleaching of Lipofectamine™ 2000 complexes containing PO-ODNs (left) or PS-ODNs (right), 4 hours after applying the complexes to the cells. The acceptor (Cy5) fluorescence ($\lambda_{\text{ex}} = 633 \text{ nm}$) and donor (rhodamine green) fluorescence ($\lambda_{\text{ex}} = 488 \text{ nm}$) are shown before (pre-bleach, A and B) and after (post-bleach, C and D) acceptor photobleaching. Detector settings and laser excitation during taking of the pre-bleach and post-bleach confocal images were kept constant. The square indicates the photobleached region. The corresponding transmission image is also shown (upper panel: Lipofectamine/PO-ODN and lower panel: Lipofectamine/PS-ODN), in which a white line is drawn around the nuclei.

CONCLUSIONS

In this paper we investigated the potential of biodegradable poly- β -aminoesters (PBAE's) as a delivery system for antisense phosphodiester and phosphothioate oligonucleotides. We showed that PBAE1 could not successfully deliver ODNs (both PO-ODNs and PS-ODNs) as the expression of ICAM-1 could not be down regulated (respectively only 0% and 20% down regulation was observed). With PBAE2 polymers, however, both the PO- and PS-ODNs down regulated the targeted ICAM-1 expression with respectively 70% and 60% at N/P ratio 10. DLS and zetapotential measurements did not point out any significant difference in the size and surface charge between PBAE1/ODN and PBAE2/ODN complexes. Also gel electrophoresis and FCS experiments revealed that the association and dissociation features of the PBAE1/ODN and PBAE2/ODN complexes are similar, thus showing no specific reason why one formulation should work better than another. A first difference that could be observed between PBAE1/ODN and PBAE2/ODN complexes was that PBAE2 offers a better protection to complexed PO-ODNs against enzymatic degradation than PBAE1. This may explain why PBAE2/PO-ODN complexes succeeded in the down regulation of ICAM-1 while PBAE1/PO-ODN complexes (but also lipofectamine/PO-ODN) failed. Another important difference was also the uptake of PBAE1/ODN and PBAE2/ODN complexes: about 30% of the cells took up the PBAE1/ODN complexes while more than 90% took up PBAE2/ODN complexes. The poor cellular uptake thus also explains the limited down regulation of ICAM-1 expression by PBAE1/PS-ODN complexes.

To further understand why PBAE2 resulted in a good antisense activity both with degradable PO-ODNs and non-degradable PS-ODNs we looked into the intracellular distribution and degradation of the delivered ODNs. Although PBAE's are considered to be biodegradable polymers that slowly release their ODNs into the cytoplasm of the cells, an enormous amount of free PO-ODNs could be observed in the nuclei of the cells very fast (i.e. in less than 4 hours) after applying the complexes to the cells. That a high amount of PO-ODNs was indeed delivered from the PBAE2 carrier became obvious from the formation of nuclear bodies in the cells, a phenomenon that only occurs when the intracellular concentration of ODNs is high enough (23). In comparison, for lipid based carriers we found that both DOTAP/DOPE liposomes and Lipofectamine™ 2000 rapidly deliver the ODNs they are carrying (7). These formulations are indeed known to rapidly escape from the endosomes thereby releasing free ODNs in the cytoplasm which then accumulate in

the nuclei (25;26). However, in opposite to PBAE2, with the lipid based carriers, nuclear bodies were only observed with PS-ODNs and not with PO-ODNs. Also, the PO-ODNs that arrived in the nuclei after release from the lipid based carriers were all degraded, in clear contrast to PO-ODNs delivered from PBAE2. As a matter of fact, the only obvious difference between the delivery of PO-ODNs from PBAE2 polymers and lipid based carriers is that the delivered PO-ODNs form nuclear bodies containing intact PO-ODNs in the case of PBAE2 polymers, while the PO-ODNs delivered by the lipid based carriers are all degraded and do not form nuclear bodies. In our opinion this results from a difference in concentration of the delivered PO-ODNs, since nuclear bodies only occur when a certain threshold of intracellular ODN concentration is reached. Therefore, the apparent higher concentration of PO-ODNs that is delivered by PBAE2 explains why not all the cytoplasmic delivered ODNs are degraded instantly, so that some of the PO-ODNs can circumvent cytoplasmic degradation and reach the nucleus in an intact way. We did indeed demonstrate previously that the amount of intracellularly delivered PO-ODNs determines the intracellular degradation time since intact intracellular PO-ODNs could only be observed when higher amount of PO-ODNs (20 μ M instead of 2 μ M) were microinjected into the cytoplasm of the cells (7). Since it was demonstrated that ODNs present in nuclear bodies can shuttle between the nucleus and the cytoplasm (where they can down regulate the expression of the target protein), the nuclear bodies formed upon delivery PO-ODNs with PBAE2 could function as a pool of intact PO-ODNs that are able to execute their antisense effect (27). Acceptor photobleaching indeed demonstrated that a fraction of the PO-ODNs in the nuclear bodies were still intact, as fluorescence resonance energy transfer could still be observed. With the lipid based carriers, the delivered concentration of PO-ODNs seems however not high enough to overcome rapid cytoplasmic degradation, and acceptor photobleaching indeed demonstrated that the PO-ODNs in the nuclei of the cells were degraded.

In conclusion, PBAE2 shows potential for the delivery of biodegradable PO-ODNs, although apparently not through a time controlled release of the ODNs from the biodegradable polymers, but rather due to the rapid release of a high concentration of ODNs that accumulate in nuclear bodies in the nuclei of the cells. These nuclear bodies contained intact ODNs, that are most likely able to shuttle between the nucleus and the cytoplasm and execute their antisense effect in this way.

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