Protection of Oligonucleotides against Enzymatic Degradation by Pegylated and Nonpegylated Branched Polyethyleneimine

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Among the cationic polymers, polyethyleneimine (PEI) is a promising candidate for delivery of oligodeoxynucleotides (ODNs). In this study, we wondered whether pegylation of PEI influences the complexation with ODNs. We especially aimed to investigate whether ODNs are differently protected against enzymatic degradation in PEI and polyethylene glycol−polyethyleneimine (PEG−PEI) polyplexes. Using fluorescence resonance energy transfer combined with fluorescence correlation spectroscopy, we found that PEI/ODN polyplexes remain to protect the ODNs they carry over a prolonged period of time while in PEG−PEI/ODN polyplexes the degradation of the ODNs slowly proceeds. We attribute this to the fact that PEI seems to compact the ODNs more firmly in the polyplexes' core than PEG−PEI, which apparently also results in a better protection against enzymatic degradation. These observations may also influence the efficiency of PEI-based ODN delivery in vivo, where pegylation is an attractive strategy to enhance the stability of the polyplexes in the blood stream.

Introduction

Currently a variety of carriers are under investigation for the delivery of nucleic acids such as plasmid DNA (pDNA). Viral vectors provide high transfection efficiencies for pDNA but suffer from the limited amount and size of the pDNA they can carry. Also they can induce severe immune responses. Nonviral vectors, such as cationic polymers[1] and cationic liposomes,[2] are advantageous over viral vectors in that they are less expensive, easier and safer to make, and more suitable for long-time storage. They can also deliver much larger pieces of pDNA when compared to viral methods.[3] Cationic lipids as well as cationic polymers spontaneously form interpolyelectrolyte complexes with the negatively charged nucleic acids. These DNA complexes are respectively called lipoplexes and polyplexes. The current transfection efficiencies of nonviral vectors are, however, far from optimal. Despite their low efficiency, nonviral vectors retain high interest due to their favorable safety profile and ease of manufacturing.

Nonviral carriers are, besides for the delivery of pDNA, also investigated for the delivery of antisense oligodeoxynucleotides (ODNs). Antisense ODNs show potential in the treatment of a wide variety of genetic and viral disorders. The principle of antisense is based on the specific inhibition of unwanted gene expression by blocking mRNA activity. This is achieved by a sequence-specific binding of the ODNs to their complementary target mRNA, which results in blocking or degradation of the target sequence. Before the ODNs can down-regulate a targeted gene expression, they have to reach the intracellular environment. The nonviral carriers should help the ODNs to enter the cells and to escape from the endosomal compartment. Generally speaking, the ODN/carrier complexes should exhibit a net positive charge to enhance the binding to the (negatively charged) cell membrane, which can be accomplished by varying the (charge) ratio of the cationic carrier to the negatively charged ODNs.[4] Also, the complexes should preferably have a small, uniform size distribution (<200 nm) to facilitate endocytosis.[5] For successful ODN delivery, the carriers should also protect the ODNs against enzymatic degradation by the nucleases in the blood and the cells.

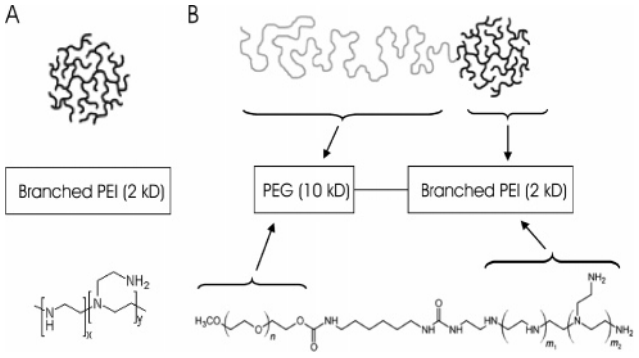

Figure 1. Schematic representation of branched PEI (A) and PEG−PEI (B) as used in this study. The molar mass of the branched PEI was 2 kDa. PEG−PEI (12 kDa) consists of one branched PEI segment (2 kDa) and one PEG segment (10 kDa).

Among the cationic polymers, polyethyleneimine (PEI) is a promising candidate for nonviral gene delivery.[6,7] The repeating unit of PEI consists of two carbon atoms followed by one nitrogen atom (Figure 1). Because of the amine groups, PEI carries a positive surface charge which allows binding and condensing of negatively charged DNA. Both the molar mass and the backbone structure of PEI (being linear or branched) influence the transfection efficiency and cytotoxicity of the PEI/DNA polyplexes.[8,9] Conflicting information is available regarding the structural requirements and the molecular weight of PEI necessary for efficient gene delivery. Kunath et al. reported that low molecular weight PEI is better for transfection than PEI with a high molecular weight.[10] In contrast, Godbey et al. found a higher transfection efficiency with increasing molecular weight.[11] Von Harpe et al. studied the transfection efficiency of a wide variety of commercially available PEIs, and although he found vast differences in transfection efficiencies, he found

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no correlation between the physicochemical properties of the PEIs and their transfection efficiency.[12]

In vitro, the use of unmodified PEI-based polyplexes comes with problems of cytotoxicity, biocompatibility, and solubility. In vivo, nonspecific protein adsorption, aggregation, and rapid blood clearance can be added to the list. Approaches for improving the solubility of PEI-based polyplexes include grafting of PEI with nonionic water-soluble polymers such as polyethylene glycol (PEG). Furthermore, this pegylation reduces the cytotoxicity and prolongs the stability and circulation of the polyplexes in the blood stream.[13,14] Also for cationic lipids, pegylation is an attractive strategy to prevent aggregation of the lipoplexes and opsonization by the reticuloendothelial system. In a previous study, we indeed found that the pegylated lipoplexes were stable over a prolonged period of time, whereas the nonpegylated lipoplexes were liable to aggregation. However, we also investigated the protection of ODNs while being complexed to nonpegylated and pegylated *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride/dioleoylphosphatidylethanolamine (DOTAP/DOPE) liposomes and found that pegylation influenced the lipoplex formation so that the ODNs remained unprotected at the surface of the lipoplexes, prone to enzymatic degradation. On the contrary, nonpegylated lipoplexes contained the ODNs on the inside, inaccessible to nuclease attack.[15]

In this study, we wondered whether pegylation of PEI influences the complexation with ODNs. To truly investigate the influence of pegylation, PEI and PEG−PEI with an identical PEI segment (branched, 2 kDa) were used (Figure 1). We especially aimed to investigate whether ODNs are differently protected against enzymatic degradation in PEI and PEG−PEI polyplexes. Following our previous work, in which we used fluorescence correlation spectroscopy (FCS) to monitor the association/dissociation between ODNs and cationic polymers and cationic liposomes,[16−20] we used FCS to study the PEI/ODN and PEG−PEI/ODN polyplexes. Also we demonstrated previously that the FCS setup allows the enzymatic degradation of fluorescently doubly labeled ODNs, both naked and complexed to cationic lipids, to be followed.[15,21,22] For details on the theoretical background of FCS, we refer to Schwille et al.[23] and Hess et al.[24] Briefly, an FCS instrument registers fluorescence intensity fluctuations caused by the diffusion of fluorescently labeled molecules through the detection volume of a microscope. From these fluorescence intensity fluctuations an autocorrelation curve can be derived, which allows calculation of the amount of fluorescent molecules in the detection volume and their diffusion coefficient. In dual-color FCS, two spectrally different fluorophores are used. The (green and red) fluorescence intensity fluctuations of the doubly labeled (green and red) molecules can then be measured simultaneously using the green and red detector of the dual-color FCS instrument. This study demonstrates for the first time that FCS allows monitoring to what extent (doubly fluorescently labeled) ODNs complexed to cationic polymers such as PEI and PEG−PEI remain stable upon exposure to nucleases.

Materials and Methods

Materials. Doubly labeled 40-mer phosphodiester ODNs (5′-GCC-GTC-TCT-GAC-TGC-TGA-TGA-CTA-CTA-TCG-TAT-AGT-GCG-G-3′, 13388.1 g/mol) were purchased from Eurogentec (Seraing, Belgium) and were purified by polyacrylamide gel electrophoresis (PAGE) by the supplier. The ODNs were doubly labeled with a rhodamine green fluorophore at the 3′ end (λ_{ex} = 488 nm, λ_{em} = 532 nm) and a Cy5 fluorophore at the 5′ end (λ_{ex} = 647 nm, λ_{em} = 670 nm).

Nonpegylated (branched) PEI (2 kDa) was purchased from Sigma (St. Louis) (Figure 1A). PEG−PEI was synthesized by the University of Nebraska Medical Center as described by Vinogradov et al., by coupling an activated PEG to the branched PEI of 2 kDa that was purchased from Sigma (Figure 1B).[25] The total content of nitrogen of the PEI and PEG−PEI was respectively 23.3 and 3.75 μmol/mg of polymer. The weight average molar mass, M_w, of PEG−PEI was determined to be 12 kDa by static light scattering (e.g., one PEG segment of 10 kDa and one branched PEI segment of 2 kDa per PEG−PEI).

DNase I (Pulmozyme, 1 unit/μL) was kindly provided by NV Roche, Brussels, Belgium. Dextran sulfate (DS) was purchased from Sigma (St. Louis). 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 μg/μL was prepared in Hepes buffer.

Preparation of PEI/ODN and PEG−PEI/ODN Polyplexes. The N/P ratio of the polyplexes is defined as the molar ratio of the total number of nitrogen atoms in the PEI segment of the polymer 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 s. Every polymer and ODN solution was prepared 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 known that the enzyme DNase I is active.[26] The polyplexes were allowed to equilibrate at room temperature for 30 min prior to use. The hydrodynamic size and ζ potential of the polyplexes were routinely checked by respectively dynamic light scattering (DLS, Malvern 4700, Malvern, Worcestershire, U.K.) and surface potential measurements (Zetasizer 2000, Malvern), as previously described.[16]

Gel Electrophoresis Measurements on the Polyplex Dispersions and ODN Degradation. To study the complexation between ODNs and PEI and PEG−PEI by agarose gel electrophoresis, 5 μL of the ODN solution (60 μg/mL) was mixed with 5 μL of a PEI and a PEG−PEI solution (the concentration of PEI and PEG−PEI being dependent on the desired N/P ratio). The polyplexes were allowed to equilibrate for 30 min 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 μL. Also 5 μL was taken from each sample and diluted with degradation buffer to a total volume of 50 μL to perform FCS measurements. Before the remaining samples were loaded onto the agarose gel, 5 μL of 50% sucrose was added.

The degradation of the ODNs, upon exposure of the polyplexes to DNase I, was studied by PAGE as follows: A 2 μL sample of the ODN solution (150 μg/mL) was mixed with 2 μL of the PEI and PEG−PEI solutions (resulting in polyplexes with a N/P ratio of 10). After equilibration, the samples were diluted with degradation buffer to a total volume of 12 μL. Then the samples were incubated with or without 0.2 unit of DNase I for 30 h. After the desired incubation time, 5 μL of 10× 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 μL of dextran sulfate (10 μg/μL) was added to release the ODNs from the polyplexes before the samples were loaded onto the polyacrylamide gel. Also from these dispersions 5 μL was removed and diluted with degradation buffer to a total volume of 50 μL to perform FCS measurements. Before the remaining samples were loaded onto the polyacrylamide gel, 5 μL of 50% sucrose was added.

FCS Measurements on the Polyplex Dispersions and ODN Degradation. Dual-color FCS measurements were performed on polyplexes composed of nonlabeled cationic polymers (PEI or PEG−PEI) and doubly labeled ODNs. A dual-color FCS setup installed on an 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

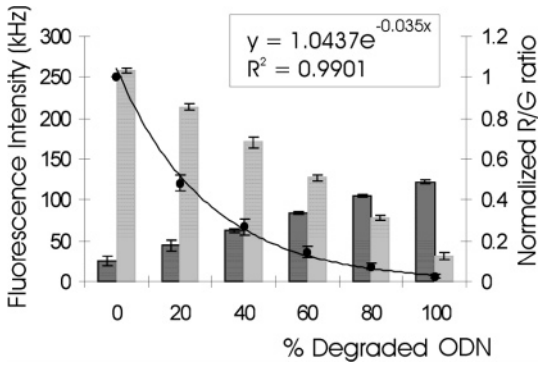

Figure 2. Green (dark gray bars) and red (light gray bars) fluorescence of solutions composed of intact and degraded doubly labeled ODNs. The total (intact + degraded) ODN concentration of all solutions was equivalent to 100 nM intact ODN. The red and green fluorescence was measured by the detectors of the FCS instrument. Laser excitation was set to 488 nm. The R/G ratio is also depicted (●). Values represent the average ± standard deviations of three independent experiments.

excitation volumes and the detection volumes optimally overlapped, the system was calibrated as described by Schwille et al.[27] The laser beam was focused at about 50 μm above the bottom of the glass-bottom 96-well plate (Grainer Bio-one, Frickenhausen, Germany), which contained the samples. The 488 and 647 nm laser beams of a krypton−argon laser (Bio-Rad, Cheshire, U.K.) were used, and the green and red fluorescence intensity fluctuations were recorded on a digital ALV 5000/E correlator while the polyplexes were excited at 488 or 647 nm during five intervals of 50 s. 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 at 488 nm.

For FCS measurements in Figure 2, 100 μL of 200 nM ODNs in degradation buffer was incubated with and without 1 unit of DNase I at 37 °C for 4 h. Then 100 μL of 2× EDTA-enriched TBE buffer was added to the Eppendorf tubes to inhibit the DNase I. Mixtures of intact and degraded ODNs were prepared by mixing 50/0, 40/10, 30/20, 20/30, 10/40, and 0/50 μL of intact/degraded ODNs. For FCS measurements in Figures 6 and 7, 50 μL samples were obtained from the gel electrophoresis experiments as described above. To follow the degradation of the complexed ODNs as a function of time (Figure 8), polyplexes were prepared by mixing 200 μL of 1 μM ODNs with 200 μL of polymer solution (respectively 0.0087 or 0.0174 μg/μL PEI and 0.054 or 0.107 μg/μL PEG−PEI to obtain a N/P ratio of 5 or 10). After 30 min of equilibration, the polyplex dispersions were diluted with degradation buffer to a total volume of 1000 μL and subsequently divided into two aliquots of 500 μL. To one 500 μL aliquot was added 1 μL (1 unit) of DNase I. The two aliquots were further divided into Eppendorf tubes each containing 50 μL of polyplex dispersion and incubated at 37 °C. After the desired incubation time, 50 μL of 2× EDTA-enriched TBE buffer was added to the Eppendorf tubes to inhibit the DNase I. On these samples, FCS measurements were performed before and after addition of 20 or 10 μL of dextran sulfate (10 μg/μL) to release the ODNs from respectively the PEI and PEG−PEI polyplexes.

Results

Fluorescence Resonance Energy Transfer (FRET) To Study the Degradation of Fluorescently Doubly Labeled ODNs. As we reported previously, in the intact ODNs used in this study FRET occurs between the rhodamine green fluorophore on the 3′ end and the Cy5 fluorophore on the 5′ end.[21] Upon excitation with 488 nm laser light, the energy is transferred from the rhodamine green (donor fluorophore) to the Cy5 (acceptor fluorophore). This turns the ODNs more red fluorescent while they become less green fluorescent. Figure 2 shows the green and red fluorescence intensities (as measured by the detectors of the FCS instrument upon excitation with 488 nm laser light) of solutions containing intact and degraded ODNs. At higher amounts of degraded ODNs, the solutions become less red fluorescent but more green fluorescent. Hence, the ratio of the red to green fluorescence (R/G ratio) decreases as a function of the amount of degraded ODNs. Indeed, degradation of the ODNs by endonucleases increases the distance between the fluorophores so that FRET no longer occurs.[21] Therefore, the R/G ratio can be used to monitor the integrity of the doubly labeled ODNs used in this study.

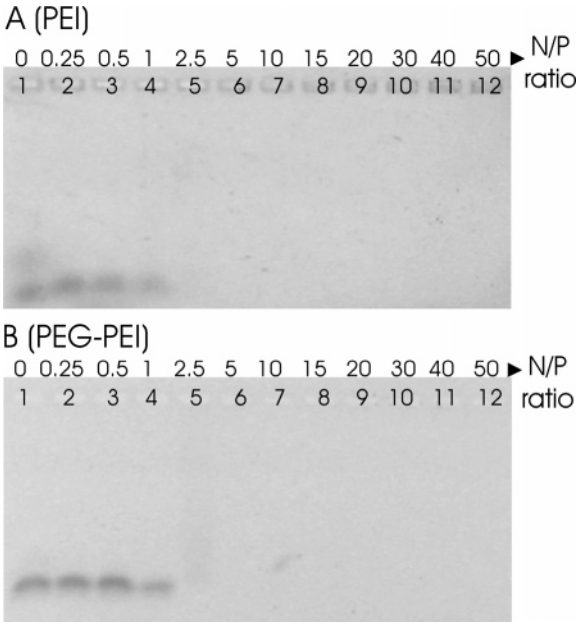

Figure 3. Gel electrophoresis on PEI/ODN (A) and PEG−PEI/ODN (B) dispersions: lane 1, free ODNs; lanes 2−12, PEI or PEG−PEI/ODN polyplexes with the N/P ratio as depicted above each lane. The amount of ODNs in each lane was 300 ng. This experiment was repeated three times with newly prepared samples, showing the same results as depicted.

Association of ODNs with PEI and PEG−PEI. The association of ODNs with PEI and PEG−PEI, as a function of the N/P ratio, was first studied by agarose gel electrophoresis. As Figure 3 shows, up to a N/P ratio of 1, free (uncomplexed) ODNs migrate in the agarose gel, thus indicating that the amount of cationic polymer is not sufficient to bind all the (negatively charged) ODNs. At a N/P ratio of 2.5 a large amount of the ODNs seem to be bound to the PEI and PEG−PEI, indicating that the amount of PEI and PEG−PEI is sufficient to bind all the ODNs.

Figure 4 shows the fluorescence fluctuation profiles of free and complexed doubly labeled ODNs as measured by FCS upon excitation at 488 nm. FRET clearly occurs in the free ODNs (Figure 4A) since the red fluorescence (acceptor fluorophore) is much higher than the green fluorescence (donor fluorophore). From the fluorescence intensities, an R/G ratio of 11.3 ± 0.6 was obtained. When PEI or PEG−PEI is added to the ODNs, the red fluorescence decreases significantly (Figure 4B,C). Also, "fluorescence peaks" are visible in the red fluorescence fluctuations of the PEG−PEI/ODN dispersions (Figure 4C), in contrast to the PEI/ODN dispersions (Figure 4B). Both observations will be interpreted and explained in the Discussion.

Dissociation of PEI/ODN and PEG−PEI/ODN Polyplexes. When negatively charged dextran sulfate is added to the

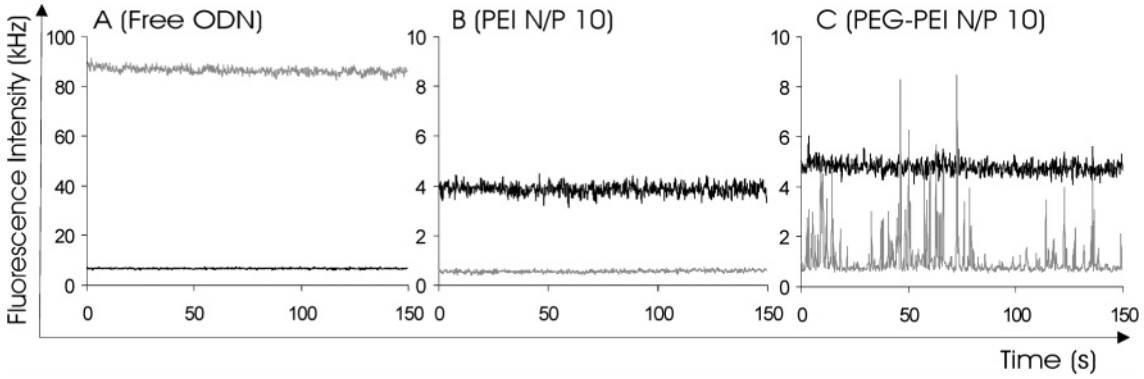

Figure 4. Representative FCS measurements on free ODNs (A) and PEI/ODN (B) and PEG−PEI/ODN (C) dispersions. The green (black line) and red (gray line) fluorescence intensity fluctuations were recorded by the FCS instrument. Laser excitation was set to 488 nm. The N/P ratio of the polyplexes was 10.

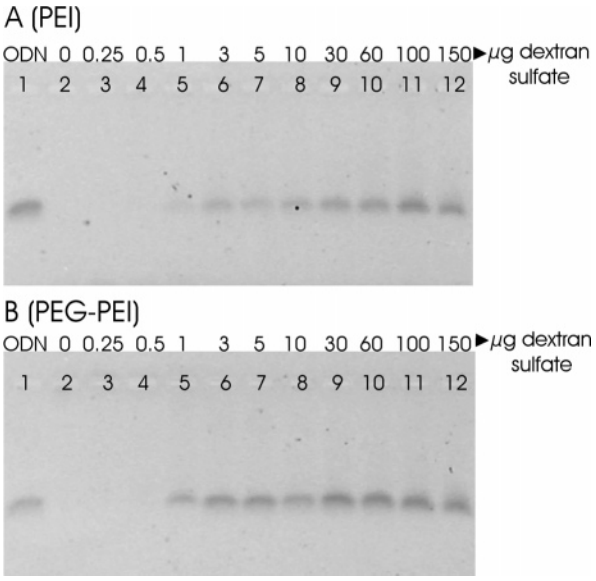

Figure 5. Gel electrophoresis on PEI/ODN (A) and PEG−PEI/ODN (B) dispersions: lane 1, free ODNs; lanes 2−12, PEI or PEG−PEI/ODN polyplexes (N/P ratio of 10) to which increasing amounts of dextran sulfate were added (as depicted above each lane in micrograms). The amount of ODNs in each lane was 300 ng. This experiment was repeated three times with newly prepared samples, showing the same results as depicted.

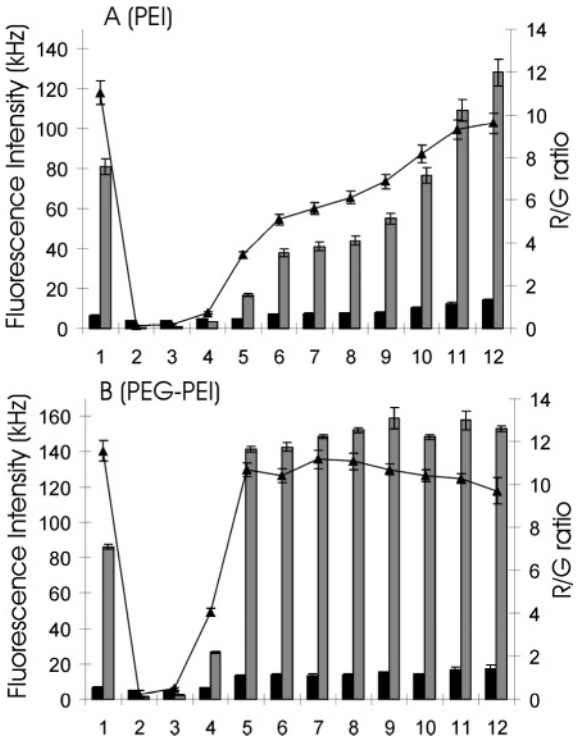

Figure 6. FCS measurements on PEI/ODN (A) and PEG−PEI/ODN (B) dispersions. The green (black bar) and red (gray bar) fluorescence intensity fluctuations were recorded by the FCS instrument. Laser excitation was set to 488 nm. The samples as measured by FCS were composed of a fraction of samples 1−12 used in Figure 5 as described in the Materials and Methods. Accordingly, lane 1 contains free ODNs, and lanes 2−12 contain PEI or PEG−PEI/ODN polyplexes (N/P ratio of 10) to which respectively 0, 0.25, 0.5, 1, 3, 5, 10, 30, 60, 100, and 150 μg of dextran sulfate were added. The R/G ratio is also depicted (▲). Values represent the average ± standard deviation of three FCS measurements per sample.

polyplex dispersions, it is expected that it will compete with the ODNs for binding to the PEI and PEG−PEI, thereby possibly displacing the ODNs from the polyplexes. As Figure 5 shows, this was indeed observed by gel electrophoresis. Release of ODNs from both PEI (Figure 5A) and PEG−PEI (Figure 5B) starts from lane 5, containing 1 μg of dextran sulfate. It can be seen, however, that a greater amount of dextran sulfate is required for complete release of the ODNs from PEI- versus PEG−PEI-based polyplexes.

The dextran sulfate-containing dispersions, as analyzed by gel electrophoresis in Figure 5, were further measured by FCS as described in the Materials and Methods. The average fluorescence intensities upon excitation of the polyplex/dextran sulfate dispersions at 488 nm, and the corresponding R/G ratio, are depicted in Figure 6. Free ODNs show higher red than green fluorescence due to FRET, which results in an R/G ratio of around 11 (Figure 6, sample 1). Upon complexation, the ODNs significantly lose their green and red fluorescence, which is due to the quenching of the fluorophores (see the Discussion). Accordingly, the R/G ratio of the complexed ODNs dropped to 0.2 ± 0.1 (Figure 6, sample 2).

Figure 6A, samples 3−12, shows the outcome of the FCS measurements on PEI/ODN dispersions containing increasing amounts of dextran sulfate. We can clearly see that the green and red fluorescence increases as a function of the amount of dextran sulfate, which indicates the gradual release of the ODNs from the polyplexes. In agreement with the gel electrophoresis experiment (Figure 5A, lane 5), the release starts from sample 5 (in Figure 6A), containing 1 μg of dextran sulfate. It takes however up to 150 μg of dextran sulfate to fully release the ODNs from the PEI/ODN polyplexes (Figure 6A, sample 12). Importantly, upon addition of increasing amounts of dextran sulfate to the PEI/ODN dispersions, not only the fluorescence

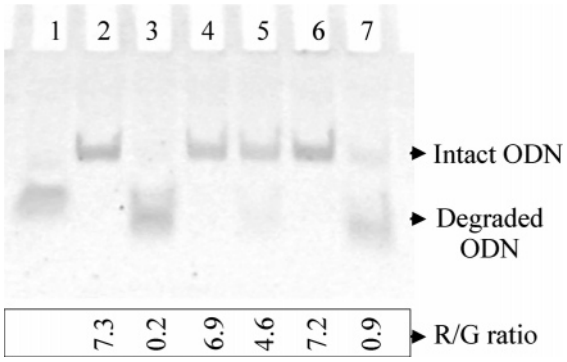

Figure 7. Gel electrophoresis on free ODNs, PEI/ODN, and PEG−PEI/ODN dispersions: lane 1, bromophenol blue marker; lanes 2 and 3, free ODNs respectively without and with 0.2 unit of DNase I; lanes 4 and 5, PEI polyplexes respectively without and with 0.2 unit of DNase I; lanes 6 and 7, PEG−PEI polyplexes respectively without and with 0.2 unit of DNase I. The N/P ratio of the polyplexes was 10. The samples were incubated at 37 °C for 30 h. Before the samples were loaded onto the gel, the DNase I activity was inhibited and the ODNs were released from the polyplexes by adding dextran sulfate. For lanes 2−7, the R/G ratio (as determined by FCS on a fraction of the samples) is also depicted. Each lane (except for lane 1) contained 300 ng of ODNs. This experiment was repeated three times with newly prepared samples, showing the same results as depicted.

intensities but also the R/G ratio gradually recovers until the value as measured for free ODNs (Figure 6A, sample 1) is reached.

As Figure 6B shows, adding dextran sulfate to PEG−PEI/ODN dispersions also increases the green and red fluorescence. The release of ODNs from the complexes starts from sample 4, containing 0.5 μg of dextran sulfate. The addition of 1 μg of dextran sulfate (Figure 6B, sample 5) releases all the ODNs, accompanied by a full recovery of the R/G ratio. This is in clear contrast to the PEI/ODN dispersions (compare with Figure 6A). Apparently, ODNs become more easily displaced from PEG−PEI/ODN polyplexes than from PEI/ODN polyplexes.

Protection of ODNs against Enzymatic Degradation by PEI and PEG−PEI. Figure 7 shows the result of polyacrylamide gel electrophoresis on free and complexed ODNs that were incubated with or without 0.2 unit of DNase I. Comparing lanes 2 and 3 shows that the degradation products run further on the gel. Lanes 4 and 6 contain respectively PEI- and PEG−PEI-based polyplexes that were incubated at 37 °C for 30 h without DNase I. After 30 h of incubation, dextran sulfate was added to release the ODNs. It seems that only intact ODNs are released from both types of polyplexes. Lanes 5 and 7 contain respectively PEI- and PEG−PEI-based polyplexes that were incubated for 30 h with DNase I. After inhibition of the DNase I and release of the ODNs by adding dextran sulfate, it can be seen that the ODNs that were complexed to PEI predominantly stayed intact, while a large fraction of the ODNs that were complexed to PEG−PEI were degraded.

The samples as analyzed by gel electrophoresis in Figure 7 were also measured by FCS. From the R/G ratio, as depicted at the bottom of Figure 7, the amount of degraded ODNs could 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 from an exponential fit ($R^2 = 0.9901$) of the normalized R/G ratio as a function of the percentage of degraded ODNs as depicted in Figure 2. For the samples in lanes 2, 4, and 6, 98 ± 2% of the ODNs remained intact. From the free ODNs in lane 3, only 0.5 ± 0.1% remained intact upon incubation with the DNase I enzyme. When the ODNs were complexed to PEI or PEG−PEI (lanes 5 and 7),

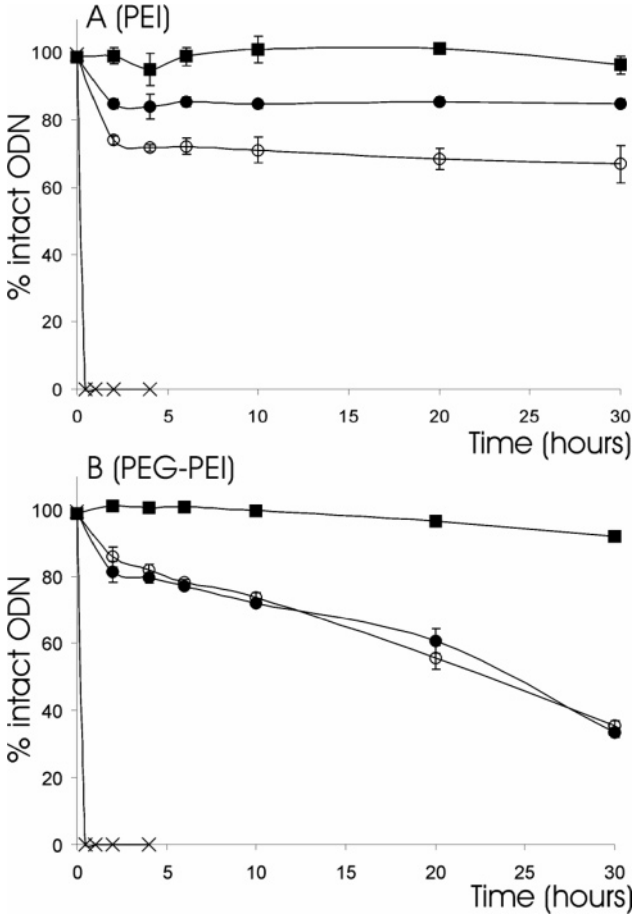

Figure 8. Percentage of ODNs remaining intact after incubation of the PEI/ODN (A) and PEG−PEI/ODN (B) polyplexes with DNase I, as measured by FCS. The percentage of intact ODNs was calculated from the R/G ratio, after inhibition of the DNase I and subsequent release of the ODNs from the polyplexes using dextran sulfate. Laser excitation was 488 nm. Key: (■) polyplexes incubated without DNase I (average ± standard deviation, N/P ratios of 5 and 10), (○) polyplexes (N/P ratio of 5) incubated with 1 unit of DNase I, (●) polyplexes (N/P ratio of 10) incubated with 1 unit of DNase I, and (×) naked ODNs incubated with 1 unit of DNase I. Values represent the average ± standard deviation of two samples per time point. When no error bars appear, they were smaller than the symbol.

respectively 82 ± 5% and 38 ± 4% of the ODNs survived the incubation with the DNase I enzyme.

We further examined the degradation kinetics of the ODNs complexed to PEI and PEG−PEI. Therefore, we measured the R/G ratio of the ODNs (upon excitation at 488 nm) released by dextran sulfate from polyplexes after being incubated with DNase I for a certain period of time. Again, from the R/G ratio, the amount of degraded ODNs was 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.

When PEI/ODN and PEG−PEI/ODN polyplexes were incubated without DNase I, the amount of intact ODNs only slightly decreased to respectively 96 ± 3% and 93 ± 1% after 30 h of incubation, indicating that almost no degradation occurred (Figure 8, squares). For PEI/ODN polyplexes with a N/P ratio of 5, 26 ± 2% of the ODNs were degraded after a couple of hours. Upon longer incubation times the degradation slowly proceeded: 33 ± 5% of the ODNs were degraded after 30 h (Figure 8A, open circles). For PEI/ODN polyplexes with a N/P ratio of 10, initially only 15 ± 1% of the ODNs became degraded while a longer incubation with DNase I did not further degrade the ODNs (Figure 8A, closed circles).

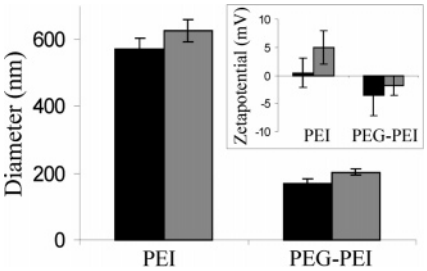

Figure 9. Hydrodynamic size and ζ potential measurements (graph inset) on PEI/ODN and PEG−PEI/ODN dispersions: (black bars) N/P ratio of 5 and (gray bars) N/P ratio of 10. Values represent the average ± standard deviation of three independent experiments.

For PEG−PEI/ODN polyplexes the protection of the ODNs seemed independent of the N/P ratio of the polyplexes (Figure 8B, open and closed circles). Initially, also 14 ± 3% of the ODNs become degraded. In contrast to PEI/ODN polyplexes (N/P = 10), more ODNs become degraded upon longer incubation with DNase I. Indeed, after 30 h 66 ± 2% of the ODNs are degraded. It should be noted that when free ODNs were incubated with DNase I under the same circumstances, complete degradation was already achieved within 30 min (Figure 8, times signs).

Hydrodynamic Size and ζ Potential of PEI/ODN and PEG−PEI/ODN Polyplexes. Figure 9 shows the hydrodynamic size and ζ potential of PEI/ODN and PEG−PEI/ODN polyplexes with N/P ratios of 5 and 10. PEG−PEI polyplexes are respectively 170 ± 13 and 203 ± 9 nm for N/P ratios of 5 and 10. PEI polyplexes are about 3 times larger, respectively 570 ± 33 and 627 ± 34 nm for N/P ratios of 5 and 10. Depending on the N/P ratio, the PEI polyplexes are neutral to slightly positive, while the PEG−PEI polyplexes are neutral to slightly negative.

Discussion

A better understanding of the physicochemical properties and biological behavior of cationic polymers is expected to provide valuable knowledge for the future design of highly efficient nucleic acid delivery systems. The in vitro transfection efficiency of cationic polymer/nucleic acid polyplexes depends, however, on a large variety of factors such as cell type, confluency of cells, the way the complexes are formed as well as their composition, the composition of the incubation medium, the time of incubation, and so on.[28] This complicates the search for optimal nucleic acid delivery systems and necessitates screening of new candidates under various experimental conditions. Physicochemical properties such as the size and charge of carrier/DNA complexes are routinely checked by respectively dynamic light scattering and ζ potential measurements. The extent to which the carriers protect the nucleic acids against enzymatic degradation is mostly investigated by agarose (in the case of pDNA) and polyacrylamide (in the case of ODNs) gel electrophoresis. This requires, however, a sufficient amount of complexes and thus a sufficient amount of cationic carriers to be able to visualize the degradation of the nucleic acids, which may be an issue for cationic carriers that are expensive or difficult to synthesize. In this study we used FCS as a tool to gain insight into the protection of ODNs against enzymatic degradation when complexed to cationic polymers. FCS is advantageous in that it can be done in very small sample volumes (μL) in the nanomolar concentration range. The low concentrations used are a major advantage when scarce DNA and polymer materials are studied. Also the small sample volume required makes the technique suitable for measurements in rare media (e.g., cytosolic lysates) and living cells.

First, we studied the association and dissociation behavior of PEI/ODN and PEG−PEI/ODN polyplexes. From a gel retardation assay (Figure 3), we observed that both PEI and PEG−PEI upon mixing with ODNs spontaneously form complexes at N/P ratios of 2.5 and higher. From gel electrophoresis, one would thus conclude that the PEG chains in PEG−PEI did not influence the complexation behavior when compared to PEI, an observation that has been reported before in the complex formation with plasmid DNA.[13] This could result from the fact that for the PEG−PEI, as used in this study, there is only about one PEG chain per cationic PEI segment (Figure 1B), thus lowering the chance that the PEG chains would shield the cationic charges on the PEI backbone.

FCS measurements, however, did reveal some differences between PEI and PEG−PEI with regard to the complexation of ODNs (compare parts B and C of Figure 4). With both PEI and PEG−PEI, a drastic decrease in the green and red fluorescence of the ODNs occurred upon complexation, which we attribute to quenching of the fluorophores due to compaction of the ODNs in the polyplexes. Probably, PEI and PEG−PEI interfere with the emitted photons through scattering or blockage (absorption) when the ODNs are present in the polyplexes' core. Clearly, electrostatic interactions between the anionic ODNs and the cationic PEI segment of PEI and PEG−PEI drive the complex formation. The polyplexes consist of a core of cationic segments (partially) neutralized by ODN strands, additionally surrounded by a shell of neutral PEG chains in the case of PEG−PEI. In the core, the ODNs are tightly packed so that the fluorophores they are carrying are quenched. The compaction of the ODNs in the core of the PEG−PEI-based polyplexes seems, however, different from that in the PEI-based polyplexes. Indeed, highly intense fluorescence peaks are visible in the red fluorescence fluctuation profile of the PEG−PEI polyplexes (Figure 4C). Such a high peak is due to movement of a PEG−PEI/ODN complex containing multiple fluorophores (and thus multiple ODNs) through the detection volume of the FCS instrument. In other words, it indicates the presence of multiple ODNs in a PEG−PEI/ODN complex. It is highly likely that PEI/ODN complexes also contain multiple ODNs. Such highly intense fluorescence peaks could, however, not be observed with PEI/ODN polyplexes (Figure 4B). We attribute this to a stronger quenching of the fluorophores in PEI-based polyplexes when compared to the PEG−PEI-based polyplexes. Assuming that the quenching of the fluorophores on the ODNs is related to the compaction of the ODNs in the polyplexes' core, PEI would thus compact the ODNs differently compared to PEG−PEI. In Figures 5 and 6 we determined the amount of dextran sulfate necessary to displace the ODNs from the polyplexes. Both gel electrophoresis and FCS measurements pointed out that the ODNs were more easily released from the PEG−PEI than from the PEI polyplexes. This observation, together with the different quenching of the fluorophores, supports the hypothesis that the ODNs are compacted more tightly by PEI than by PEG−PEI.

In a next set of experiments we studied the degradation of the ODNs complexed to PEI and PEG−PEI upon dispersion of the polyplexes in a DNase I solution. The fluorescently doubly labeled ODNs used in this study show FRET: degradation of the ODNs increases the green fluorescence and lowers the red fluorescence. Consequently, a high R/G ratio indicates intact ODNs, while a low one points out degradation of the ODNs.[21] On one hand, for a solution of free ODNs the R/G ratio is

representative of the amount of intact free ODNs (e.g., Figure 2). On the other hand, upon complexation of the ODNs with PEI and PEG−PEI, the fluorescence drops to nearly zero. Due to this severe quenching, the R/G ratio of the complexed ODNs is thus not representative of the amount of intact ODNs in the polyplexes. Figure 6 showed that the R/G ratio of the ODNs, released from the polyplexes, is restored to its original level (provided the ODNs were not degraded). Also, when the ODNs are free in solution, it is known that FRET can only occur between the labeled ends of a single ODN, while, with greater compaction in the polyplexes, it is possible that FRET occurs between fluorophores of different ODNs. Therefore, the R/G ratio of the ODNs was only interpreted upon release of the ODNs from the polyplexes. In this case, the R/G ratio reflects to what extent the ODNs were protected against enzymatic degradation while they were present in the polyplexes. The ODNs were released from the polyplexes by adding the negatively charged dextran sulfate. It should be noted that at high amounts of dextran sulfate the green and red fluorescences of the ODNs released from the polyplexes (e.g., sample 12 in Figure 6) are higher than the green and red fluorescences of the ODN solution before complexation (sample 1 in Figure 6). This increase in fluorescence could not be attributed to the nonfluorescing dextran sulfate, nor were the fluorescence properties of the ODNs altered in the presence of dextran sulfate. Instead, we found that the amount of ODNs in the solutions containing dextran sulfate had increased, indicating that dextran sulfate also displaced the ODNs that sticked to the bottom of the wells. The R/G ratio is, however, not dependent on the concentration of the ODNs in solution, explaining why the R/G ratios before and after complexation are similar. Therefore, the increased fluorescence does not impede the use of the R/G ratio to monitor the degradation of the ODNs as a function of time.

Let us now focus on the outcome of the FCS measurements on polyplexes with a N/P ratio of 10 in Figure 8 (closed circles). Initially, PEI and PEG−PEI equally protect the ODNs they carry against enzymatic degradation: 15 ± 3% of the ODNs degrade during the first 2 h. One could wonder whether these degraded ODNs originate from free (uncomplexed) ODNs present in the dispersions, indicating that 15 ± 3% of the ODNs remained free in solution. However, Figure 3 clearly shows that no free ODNs appear at a N/P ratio of 5 or 10. Therefore, the initial degradation as observed in Figure 8 is highly likely attributed to the enzymatic degradation of ODNs while being complexed to PEI and PEG−PEI. We mentioned before that the complexed ODNs are present in the polyionic core of the polyplexes. It seems that, for the polyplexes with a N/P ratio of 10, 15 ± 3% of the ODNs are still accessible to nucleases, probably as a result of being located at the edge of the polyplexes' core. By analogy, free regions of pDNA strands have been observed to dangle from the core of PEI/DNA polyplexes.[29]

Figure 8 clearly shows that pegylation of the PEI-based nanoparticles has an influence on the protection of the ODNs: PEI/ODN polyplexes remain to protect the ODNs they carry over a prolonged period of time, while in PEG−PEI/ODN polyplexes the degradation of the ODNs slowly proceeds (reaching 66 ± 2% of degraded ODNs after 30 h of incubation). As reported above, PEI seems to compact the ODNs more firm in the polyplexes' core than PEG−PEI. This firm compaction apparently also results in a better protection against enzymatic degradation. Alternatively, PEG−PEI polyplexes exhibit a core−shell structure in which the polyplex "core" is shielded from the environment by a "shell" of hydrophilic PEG chains.[30] This PEG shield may provide an additional (steric) barrier for DNase attack and may thus contribute to the protection of the ODNs. The latter is however not supported by the results in this study. A possible explanation for the worse protection of ODNs in PEG−PEI polyplexes is the following. In essence, polyplexes are oppositely charged polyion strands held together by electrostatic interactions. It is expected that polyplexes are subjected to dynamic processes of binding and dissociation of the involved polyelectrolytes. During these "rearrangements", ODNs from the inner part of the polyplexes' core could relocate to the edge of the polyplexes' core, becoming accessible for enzymatic degradation. Since weaker polyelectrolyte interactions seem to exist in the PEG−PEI polyplexes (as indicated by the easier displacement of the ODNs by dextran sulfate), the rearrangements of the ODNs in the PEG−PEI/ODN polyplexes may be more pronounced (when compared to those in PEI/ODN polyplexes), leading to more opportunities for DNase I attack. Since the ODNs are about 13.3 kDa and the PEI polymers are about 2 kDa, one would expect that 6−7 PEI polymers can bind to a single ODN. The ODN-binding PEI segment of the PEG−PEI polymers is also 2 kDa, but the PEG chains (10 kDa) could make it more difficult for 6−7 PEG−PEI polymers to bind to a single ODN. This could explain why the ODNs more easily "rearrange" in the PEG−PEI-based polyplexes. Another difference between the PEI- and PEG−PEI-based polyplexes is the size of the particles, with PEG−PEI polyplexes being about 3 times smaller than the PEI polyplexes. This implies that the effective surface area for DNase I attack is larger for the smaller PEG−PEI polyplexes when compared to the larger PEI polyplexes, which could contribute to the more pronounced degradation in the PEG−PEI polyplexes. It should be noted that free ODNs were fully degraded within the first 30 min of incubation with the DNase I. From this point of view, one should stress that both PEI and PEG−PEI offer a drastic improvement in the lifetime of the complexed ODNs.

The degradation of the ODNs was also followed by PAGE (Figure 7). When analyzed by gel electrophoresis (using Image J), 12% and 72% of the ODNs were degraded in respectively PEI and PEG−PEI polyplexes after incubation for 30 h with DNase I. When the degradation was calculated from the R/G ratio of the same samples that were analyzed by FCS, these values were respectively 18 ± 5% and 62 ± 4% at the 30 h time point. These values agree very well with the degradation that was observed in Figure 8: respectively 15 ± 3% and 66 ± 2% of the ODNs were degraded after incubation of PEI and PEG−PEI polyplexes with DNase I for 30 h. This points out that FCS is a valuable alternative for gel electrophoresis to follow the degradation of ODNs complexed to cationic carriers.

In the literature, there is only one report comparing the stability of ODNs complexed to PEI and PEG−PEI. By densitometric analysis of PAGE gels, Brus et al. found that PEI and PEG−PEI (with molar mass ranging from 0.8 to 2 kDa) equally protected the complexed ODNs with a recovery of 90% of intact ODNs.[31] The shorter incubation time (2 h), lower DNase I concentration (e.g., for free ODNs, only 50% degraded under these circumstances), and different N/P ratio (N/P of 20) most likely explain why the degradation was less pronounced when compared to our observations. They also found that PEG−PEI polyplexes with more, but shorter, PEG chains protected more efficiently when compared to polyplexes containing fewer PEG chains of a higher molecular weight. Godbey et al. found, using gel electrophoresis, that plasmid DNA that was complexed with PEI (branched, 25 kDa) was not degraded when exposed to at least 25 units of DNase I for 24 h.[32] With regard to other polymers, Harada et al. used capillary gel electrophoresis to

determine the protective properties of PEG−pLL (pegylated poly-L-lysine) polyplexes and found that after 2 h of incubation with DNase I 60% and 85% of the ODNs were still intact, dependent on the density of the PEG shell.[33] Clearly, these studies demonstrate that the stability of the complexed ODNs depends on the polyplexes' characteristics such as the density of the PEG shell. Nevertheless, Fisher et al. found that in vivo pegylation of PEI worsened the protection of the ODNs after intravenous injection of the polyplexes.[14] Also the pegylated PEI polyplexes used in this study did not offer a better protection than the unpegylated PEI polyplexes, which may suggest that PEG−PEI did not result in a sufficiently dense PEG shield to prevent nuclease attack.

The complexation between oligonucleotides[17] and plasmid DNA[34,35] with PEI and PEG−PEI has been studied by FCS before, both in buffer and in living cells. This paper is, however, the first report of a study that uses FCS to obtain more information on the protection that PEI and PEG−PEI polymers offer to complexed ODNs. Since PEI and PEG−PEI with an identical PEI segment (branched, 2 kDa) were used (Figure 1), we could truly investigate the effect of pegylation. In conclusion, pegylation lowered the protective properties of the PEI/ODN polyplexes, especially at longer incubation times, an observation which may be related to the weaker compaction of the ODNs with the PEG−PEI polymers. These observations may also influence the efficiency of PEI-based ODN delivery in vivo, where pegylation is an attractive strategy to enhance the stability of the polyplexes in the blood stream.

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