Studying Pegylated DNA Complexes by Dual Color Fluorescence Fluctuation Spectroscopy

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ABSTRACT: In this study, dual color fluorescence fluctuation spectroscopy (dual color FFS) was explored to characterize the association of oligonucleotides (ONs) to pegylated cationic block copolymers, both diblock poly(ethylene glycol)−poly(ethylenimine) (pEG-pEI) and multiblock pEG-pEI. The resulting polyelectrolyte complexes consist of a core of polyion strands, surrounded by a shell of pEG chains. To study the association of the ONs to pEG-pEI by dual color FFS, the oligonucleotides and the cationic block copolymers were labeled with spectrally different fluorescent dyes. Two alternative approaches were executed to label the pEG-pEI's: the dye was attached either to the pEG segment or to the cationic pEI segment of pEG-pEI. When the (diblock and multiblock) pEG-pEI's were labeled on the pEG segment, dual color FFS on the doubly labeled FITC-pEG-pEI/Cy5-ONs complexes revealed the simultaneous appearance of peaks of high fluorescence intensity in both detector channels. This indicates that numerous Cy5-ONs and numerous FITC-pEG-pEI chains move together through the detection volume of the FFS instrument, proving the interaction between many Cy5-ONs and many FITC-pEG-pEI strands to form one multimolecular complex. However, when the fluorescent dye was bound to the cationic pEI segment of diblock or multiblock pEG-pEI, these simultaneously occurring peaks were never observed. Gel electrophoresis experiments proved that diblock pEG-pEI labeled at the pEI segment could not complex the ONs anymore. Multiblock pEG-pEI labeled at the pEI segment was still able to fully complex the oligonucleotides (as shown by gel electrophoresis). The absence of peaks of high fluorescence intensity in FFS measurements was attributed to the quenching of the fluorophores in the core of the complexes. From these results, it could be concluded that dual color FFS allows studying the association and dissociation of pEG-pEI/ONs provided that the fluorescent label of the polymer is attached to the pEG segment and not to the pEI segment.

Introduction

Gene and antisense therapy offer a straightforward approach to cure both acquired and inherited disorders. They may offer promising alternatives to traditional symptomatic therapies for cancer and diseases of viral, inflammatory, or genetic origin.[1,2] Gene therapy may eventually allow adding, correcting, or replacing genes. In antisense therapy, oligonucleotides are delivered to specifically inhibit gene expression by blocking translation, splicing, or transcription processes. However, these therapeutic macromolecules are strongly dependent on pharmaceutical carriers to cross the barriers of cellular uptake, endosomal escape, stability, and (in the case of gene therapy) migration to the nucleus.

Cationic lipids and cationic polymers are investigated as nonviral pharmaceutical carriers for DNA.[3,4] Cationic lipids as well as cationic polymers spontaneously form soluble interpolyelectrolyte complexes with the negatively charged nucleic acids. These DNA complexes are called respectively lipoplexes and polyplexes.[5] Pharmaceutical carriers bearing hydrophilic segments (such as poly(ethylene glycol), pEG) were developed to stabilize and prolong the circulation lifetime of nucleic acid-containing particles and to improve targeting strategies.[6−8] These "pegylated carriers" result in DNA complexes consisting of a more or less hydrophobic core of partially neutralized polyion strands, surrounded by a shell of hydrophilic chains. Figure 1 represents a schematic view of oligonucleotides complexed to a cationic homopolymer and a cationic block copolymer, respectively resulting in a "nonstructured" DNA complex and a more ordered "core−shell" DNA complex. Nonstructured DNA complexes require a substantial excess of cationic lipid/polymer over ONs to prevent spontaneous aggregation of the DNA complexes. However, such positively charged particles are prone to nonspecific interactions with plasma proteins and negatively charged cell surfaces. This can result in aggregation or dissociation of the DNA complexes, activation of the complement system, rapid clearance of the DNA particles by phagocytes, and aspecific binding with nontarget cells. The shell of electroneutral, hydrophilic chains of core−shell DNA particles can avoid these problems by reducing the interactions with biological macromolecules and cell surfaces.

To obtain breakthroughs in gene and antisense delivery, there is an urgent need to study the biophysical behavior of DNA complexes in cells. Fluorescence fluctuation spectroscopy (FFS), which can be applied on a cellular scale,[9] shows potential for that purpose. As outlined in Figure 2, FFS basically monitors fluorescence intensity fluctuations in the focal volume of a microscope. The fluorescence fluctuations are due to the diffusion of fluorescent molecules in and out of the focal volume. In dual color FFS, both interacting components are labeled with spectrally different fluorophores (e.g., red and green), and their emission light is detected separately by two detectors monitoring the same focal volume (Figure 2). When dissociated, the components, which diffuse in and out of the focal volume, are only

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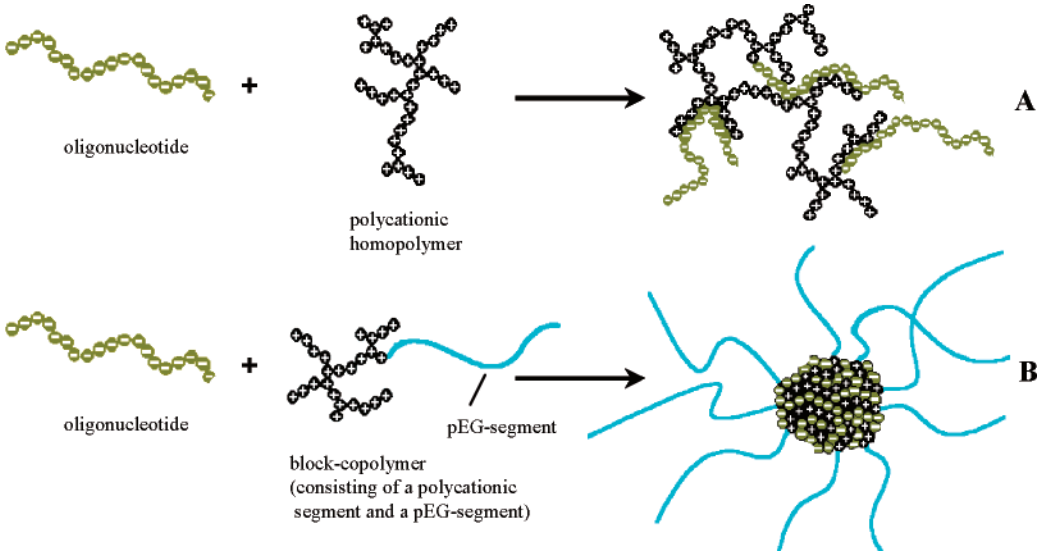

Figure 1. Schematic representation of the formation of "nonstructured" DNA complexes (A) and of DNA complexes with a "core−shell" architecture (B).

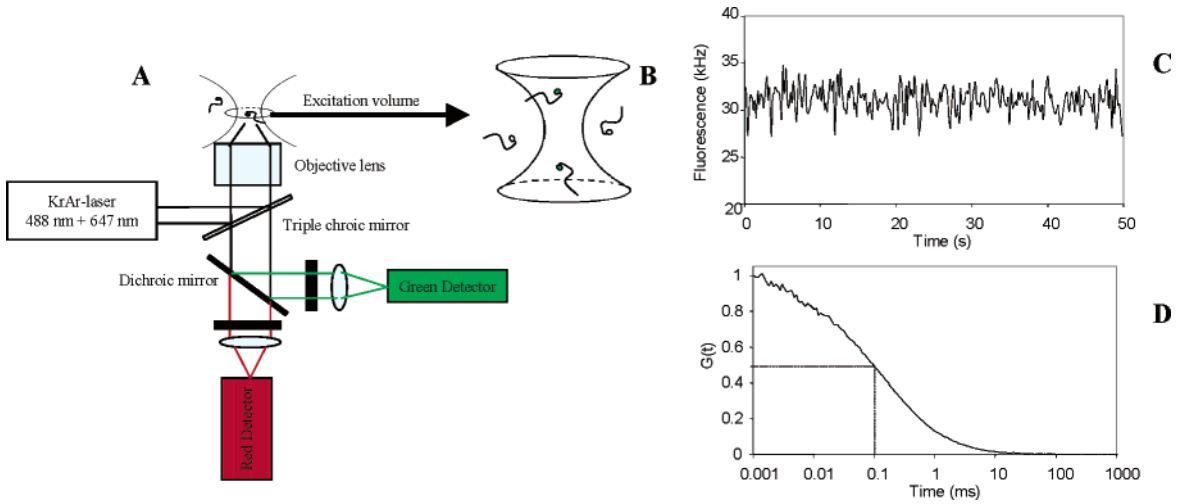

Figure 2. (A) Schematic setup of a FFS instrument. The excitation light from the laser is reflected by a triple chroic mirror into the rear aperture of the objective lens. The light emitted by the fluorescent molecules in the focal volume passes through the objective lens and the triple chroic mirror, to be split by the subsequent dichroic mirror into a red and a green component. Schematic view (B), fluorescence intensity profile (C), and corresponding autocorrelation curve (D) of fluorescent-labeled ONs, freely migrating through the focal volume. Inside the focal volume the fluorophores are excited and emit light (filled circles in B), and outside the focal volume the fluorophores remain in a darkened state (open circles in B).

detected in one detector, as they carry a red or a green fluorophore. However, when associated, the complex, which diffuses in and out of the focal volume, is detected in both detectors simultaneously because it bears both red and green fluorophores.

Our former work focused on the complexation of ONs with cationic homopolymers (e.g., polylysine), resulting in nonstructured polyplexes.[10−13] Especially, we showed that both single and dual color FFS allow studying the association and induced dissociation of oligonucleotides and cationic polymers. Others applied (single color) FFS to study the condensation of plasmid DNA with cationic carriers.[14,15] In this paper we investigate whether dual color FFS also allows evaluating the association/dissociation behavior of pegylated DNA complexes. Two distinct types of pegylated polycations were investigated: a diblock copolymer and a multiblock copolymer (Figure 3), equal in chemical formula but of different molecular weight and degree of pegylation. Two distinct approaches were used to fluorescent label these copolymers: the fluorophore was attached either to the cationic segment or to the distal end of the pEG strand of the copolymer.

Materials and Methods

Oligonucleotides. The 20-mer phosphodiester ONs (5′-CCC-CCA-CCA-CTT-CCC-CTC-TC-3′) (molar mass 5830 g/mol), 25-mer phosphodiester ONs (5′-TCT-GGG-TCA-TCT-TTT-CAC-GGT-TGG-C-3′) (molar mass 8183 g/mol), and labeled analogues (using rhodamine green (Rh) or Cy5 as fluorescent marker) were synthesized by Eurogentec (Seraing, Belgium). The fluorescent labeling occurred at the 5′ end of the ONs: each oligonucleotide contained one label (Rh or Cy5). The concentration of the ONs stock solutions (in 20 mM Hepes buffer at pH 7.4) was determined by absorption measurements at 260 nm (1 OD_{260} = 33 μg of ONs/mL). The contribution to the absorption at 260 nm by the label was taken into account in the determination of the concentration of the labeled ONs. The ONs stock solutions were further diluted with Hepes buffer.

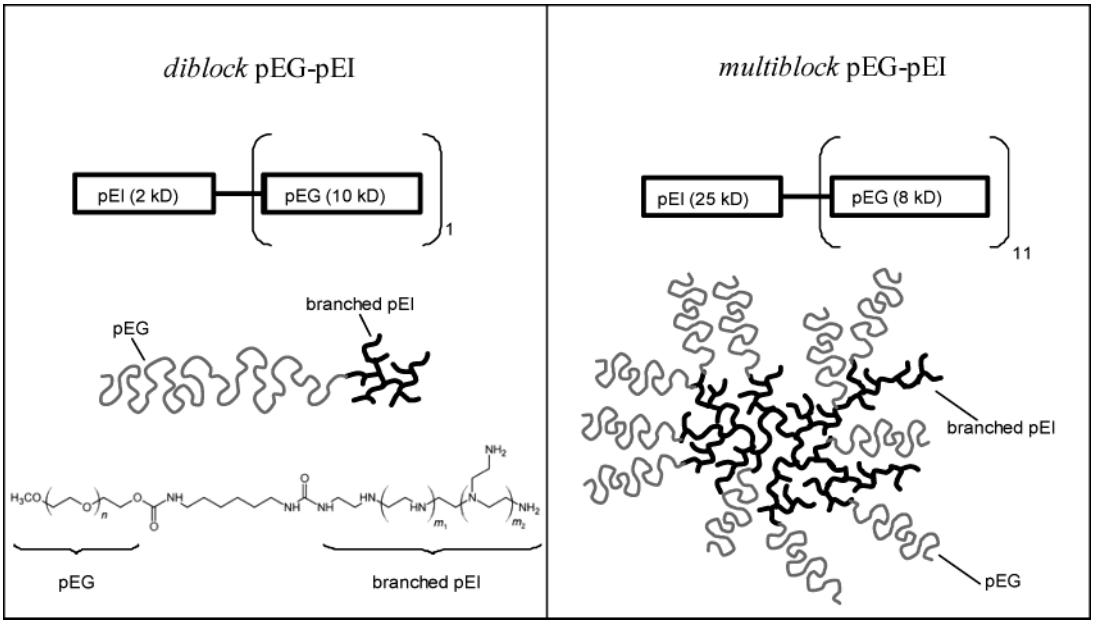

Figure 3. Schematic representation of diblock and multiblock pEG-pEI. The diblock pEG-pEI (batch PI20100, MW 12kD) consists of one branched pEI segment (2 kDa) and one pEG segment (10 kDa). The multiblock pEG-pEI (batch PI25080, MW 113 kDa) consists of one branched pEI segment (25 kDa) onto which 11 pEG segments (8 kDa each) are coupled.

Polymers. The diblock and multiblock poly(ethylene glycol)−poly(ethylenimine) (pEG-pEI) copolymers (Figure 3) were synthesized by Prof. Dr. S. Vinogradov (University of Nebraska).[16] The average molecular weight (MW), as determined from static light scattering, was respectively 12 kDa (for the diblock, batch PI20100) and 113 kDa (for the multiblock, batch PI25080). The total content of nitrogen was respectively 3.75 and 4.92 μmol/mg of polymer.[16] pEG-pEI stock solutions were prepared in Hepes buffer.

Branched pEI (nonpegylated) was purchased from Sigma (St. Louis, MO). The molecular weight of the two batches was respectively 2 and 25 kDa. Prior to use, the polymer was desalted over a Sephadex-G25 column (ϕ 10 × 100 mm) in Hepes buffer. The polymer concentration was determined finding the amine concentration using the method of Snyder and Sobocinski.[17]

FITC-pEG-*N*-hydroxysuccinimide (FITC-pEG-NHS, MW 5kDa) was purchased from Shearwater Corp. (Huntsville, AL). Purification over a Sephadex-G25 column (ϕ 10 × 100 mm) showed that no excess of free FITC could be detected by the absorbance detector (Waters 486, Millipore Corp., Milford, MA).

Fluorescent Labeling of the pEI Segment in Diblock and Multiblock pEG-pEI. As outlined in the Introduction, two types of fluorescent pEG-pEI were investigated. While in the first type the pEI segment was fluorescent-labeled, in the second type a fluorophore was attached to the distal end of the pEG segment (see below).

The pEI segment of both diblock and multiblock pEG-pEI was labeled using a succinimidyl ester-activated Cy5 dye (Cy5-SE) (FluoroLink Cy5 monofunctional dye, Amersham Pharmacia, Piscataway, NJ), following the manufacturer's procedure. The resulting fluorescent polymers were termed respectively diblock and multiblock pEG-pEI-Cy5. The diblock pEG-pEI-Cy5 was purified on a Sephadex-G25 column (ϕ 10 × 100 mm), which was previously equilibrated with Hepes buffer. The fractions containing fluorescent pEG-pEI-Cy5 were collected. From the determination of both the amine concentration (using the method of Snyder and Sobocinski[17]) and the absorbance of the label, the average number of labels attached to the polymer was obtained. It could be calculated that, on the average, the pEG-pEI-Cy5 chains bore one Cy5 label. The multiblock pEG-pEI-Cy5 was purified via dialysis (molecular weight cutoff 12−14 kDa; Medicell International Ltd.). On the average, one fluorophore per copolymer was detected.

Preparation of FITC-pEG-pEI. To obtain a diblock pEG-pEI which is fluorescent-labeled with FITC at the pEG segment (diblock FITC-pEG-pEI), equal volumes of pEI (MW 2Da) and FITC-pEG-NHS (MW 5 kDa) stock solutions (in 0.2 M $NaHCO_3$ at pH 9.3) were mixed, yielding equal molar concentrations in the reaction mixture. After incubation for 1 h, unreacted pEI was removed using a Sephadex-G50 column (ϕ 10 × 300 mm) and 20 mM Hepes buffer (pH 7.4) as mobile phase. Nonconjugated FITC-pEG was removed by means of cation exchange chromatography using a carboxymethyl-Sephadex column (ϕ 10 × 40 mm, loading buffer: 20 mM Hepes pH 7.4, elution buffer: 20 mM Tris pH 9 with 0.7 M NaCl), followed by desalting over a Sephadex-G50 column (ϕ 10 × 100 mm) in Hepes buffer. Finding the amine concentration using the method of Snyder and Sobocinski[17] and measurement of the absorbance of the label allowed the determination of the average number of FITC-pEG chains conjugated to one pEI chain. It could be calculated that one pEI chain was complexed to one FITC-pEG strand. The resulting diblock FITC-pEG-pEI copolymer (7 kDa) served as a labeled model for the diblock pEG-pEI copolymer (12 kDa, batch PI20100).

To create a labeled equivalent of the multiblock pEG-pEI (113 kDa, batch PI25080), a dispersion of the multiblock pEG-pEI was mixed with an equal molar amount of FITC-pEG-NHS (as described above for the fluorescent labeling of the diblock pEG-pEI) and further purified via dialysis (molecular weight cutoff 12−14 kDa; Medicell International Ltd.).

Preparation of Polyplexes. The N/P ratio of the polyplexes is defined as the molar ratio of the total number of nitrogen atoms of the polymer to the number of DNA phosphates. The polymer/ONs complexes (varying in N/P ratio) were prepared by adding (in one step) different volumes of the polymer stock solution to a fixed volume of the ONs stock solution, followed by vortexing for 10 s. To obtain the final ONs concentration of 10 μg/mL (1.7μM), the dispersions were further diluted with Hepes buffer. The polyplexes were allowed to equilibrate for 30 min at room temperature prior to use.

For FFS measurements on the polymer/ONs complexes, 100 μL of the dispersion was prepared as described above; however, the final ONs concentration equaled 0.2 μg/mL (34 nM). After preparation, 50 μL of the sample was immediately transferred into a 96-well plate (Grainer Bio-one, Frickenhausen, Germany) to begin the FFS measurements.

Agarose Gel Electrophoresis. Thirty minutes after the preparation of the polyplex dispersions, 30 μL of these disper-

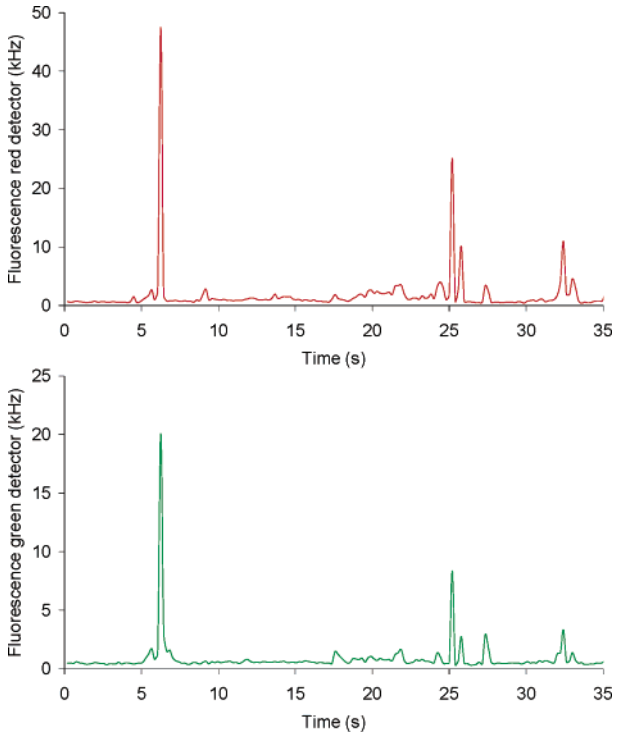

Figure 4. Fluorescence fluctuation profiles of Tetraspeck beads as simultaneously registered by the FFS "red detector" (upper panel) and "green detector" (lower panel).

sions (containing 20 μg/mL Rh-labeled ONs) was mixed with 5 μL of a 50% sucrose solution in distilled water and placed in the wells of a 1.1% agarose gel. A TBE buffer was used containing 10.8 g/L Tris base, 5.5 g/L boric acid, and 0.58 g/L EDTA. A potential of 100 V was applied for 60 min. The oligonucleotides in the gel were detected on the basis of the fluorescence of their Rh label.

Spectrofluorimetry. The fluorescence of polymer/Rh-ONs complexes was measured on an SLM-Aminco Bowman spectrofluorimeter (SLM-Aminco Bowman; $\lambda_{ex} = 488$ nm, $\lambda_{em} = 510$ nm), 30 min after their preparation. The fluorescence of each dispersion was measured three times.

Particle Size and Zeta-Potential Measurements. Dynamic light scattering (DLS) and zeta-potential (ζ) measurements on the polyplexes were respectively carried out on a Malvern 4700 instrument (Malvern, Worcestershire, UK) and a Malvern Zetasizer 2000 (Malvern, Worcestershire, UK), as previously described.[13]

Fluorescence Fluctuation Spectroscopy (FFS). Dual color FFS experiments were performed on a dual color FFS setup installed on a MRC1024 Bio-Rad confocal laser-scanning microscope. 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). The laser beam was focused at about 100 μm above the bottom of the glass-bottom 96-well plate (Grainer Bio-one, Frickenhausen, Germany), which contained the samples. Prior to use, the thickness of the bottom of the 96-well plate was measured using a micrometer, and the collar rim correction of the lens was adjusted for. The 488 and 647 nm laser beams of a krypton−argon laser (Biorad, Cheshire, UK) were used. The intensities of both excitation wavelengths were controlled independently from each other, using an acousto optic tunable filter. (Opto-Electronique, St. Rémy Les Chevreuse, France). To check whether the detectors adequately detected the fluorescence fluctuations in the focal volumes (i.e., to verify whether the excitation volumes and the detection volumes optimally overlapped), the system was optimized as described by Schwille et al.[18] The size of the detection volume was calculated from autocorrelation measurements with Rh and Cy5 solutions.

A picomolar dispersion of Tetraspeck beads (90 nm diameter, Molecular Probes, Eugene, OR) was used as a positive control sample. These polystyrene beads contained four different kinds of fluorophores with the excitation/emission wavelengths 365/430, 505/515, 560/580, and 660/680. Simultaneous excitation with 488 and 647 nm resulted in the simultaneous appearance of "highly intense fluorescence peaks" for both detector channels (Figure 4). A clear proof that both detectors simultaneously noticed when a highly fluorescent, multicolored particle was migrating through the focal volume.

Each polymer/ONs dispersion was independently prepared three times, and at least 20 FFS measurements were done on each sample. In each FFS experiment, the fluorescence fluctuations were measured for 30 s. "Highly intense fluorescence peaks" (i.e., bursts of high fluorescence intensity) in the fluorescence fluctuation profiles were identified by a statistical approach previously described.[10,19] Hereby a threshold level is calculated, above which all fluorescence values are identified as "highly intense fluorescence peaks". In the absence of highly intense fluorescence peaks (i.e., when no threshold level could be calculated), the fluorescence fluctuations were analyzed by autocorrelation analysis, and a diffusion coefficient could be calculated (as described elsewhere).[13]

Results and Discussion

The electrostatic interactions between the anionic ONs and the cationic pEI segment in pEG-pEI are responsible for the complex formation. The hydrophilic pEG strands are neutrally charged and indifferent to these interactions. The spontaneously formed complexes consist of a more or less hydrophobic core of partially neutralized polyion strands, surrounded by a shell of neutral, hydrophilic pEG chains. Indeed, because of this pEG shell, the ζ potential of the complexes equaled zero (data not shown). Even at higher N/P ratios (N/P $\gg$ 8) the ζ potential remained zero, when both the diblock and the multiblock pEG-pEI were used. Dynamic light scattering showed that the hydrodynamic size of the complexes was around 200 nm (data not shown).[12] Even at N/P ratios approaching electroneutrality, the size did not change, indicating that the pEG shell prevents aggregation of the neutral DNA complexes.

Figure 5A shows that upon complexation of Rh-ONs with pEG-pEI the fluorescence decreases, presumably because of (self-)quenching of the fluorophores since the local concentration of labeled ONs in the complex is much larger than for Rh-ONs free in solution. However, although both diblock and multiblock pEG-pEI form core−shell-type polyplexes, the quenching of the Rh-ONs is different. At higher N/P ratios, the fluorescence remains quenched for Rh-ONs complexed to the diblock pEG-pEI whereas there is a partial recovery of the fluorescence in cases concerning the multiblock pEG-pEI. Gel electrophoresis shows that for both types of polyplexes no free 25-mer Rh-ONs are present in the dispersion at N/P ratio higher than 8, which would also increase the fluorescence. Therefore, the recovery of the fluorescence intensity for multiblock pEG-pEI at higher N/P ratio has to be related to less (self-)quenching between the Rh labels due to an altered distribution of the Rh-ONs in the complexes.[10,12] The discrepancy between both types of pEG-pEI is not due to their different degree of pegylation but to the differences in their cationic segments since the same discrepancy is observed for Rh-ONs complexed to the (nonpegylated) pEI homopolymer of respectively 2 and 25 kDa (Figure 5B).

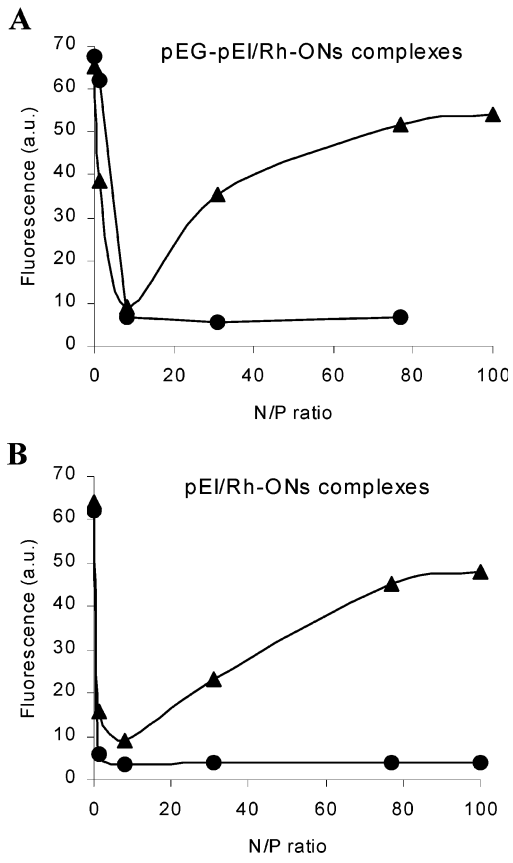

Figure 5. Fluorescence of polymer/ONs complexes (differing in N/P ratios) as measured with a conventional fluorimeter: (A) Rh-ONs (25-mer) complexed to pEG-pEI (diblock, ●; multiblock, ♦) and (B) Rh-ONs (25-mer) complexed to (non-pegylated) pEI (2 kDa, ●; 25 kDa, ♦). Each dispersion was measured three times.

Gel electrophoresis experiments revealed another difference in complexation behavior of diblock and multiblock pEG-pEI. Figure 6 shows that diblock pEG-pEI is not able to fully complex 20-mer Rh-ONs as a smear can be observed in lanes 2−4. It indicates that a major part of the Rh-ONs are still free which enables them to migrate toward the anode. However, multiblock pEG-pEI is able to complex the 20-mer Rh-ONs completely (lanes 5−7). On the other hand, both diblock and multiblock pEG-pEI are able to fully complex *25*-mer Rh-ONs (lanes 9−11 and lanes 12−14). As for (non-pegylated) pEI carriers, both 2 kDa pEI and 25 kDa pEI are able to fully complex both the 25-mer and the 20-mer Rh-ONs (data not shown). Gel electrophoresis experiments on diblock and multiblock FITC-pEG-pEI analogues (data not shown) revealed a complexation behavior identical to the unlabeled pEG-pEI shown in Figure 6.

Figure 7C,D shows the fluorescence fluctuations of a diblock FITC-pEG-pEI/Cy5-ONs dispersion measured at the same time by the red (7C) and green (7D) detector of the FFS setup. Compared with the fluorescence fluctuations of free Cy5-ONs (Figure 7A) and free diblock FITC-pEG-pEI copolymer (Figure 7B), three major observations are made. First, highly intense fluorescence peaks appear in the fluorescence fluctuation profile. This can be explained as follows. When a complex exists of many fluorescent-labeled molecules, its fluorescence becomes much higher than that of a free molecule. Consequently, when such a complex comes into the focal volume, a highly intense fluorescence peak appears in the fluorescence fluctuation profile. Second, the average background fluorescence intensity, representing the average number of fluorescent molecules in the focal volume, significantly decreases upon complexation (compare Figure 7C,D with respectively Figure 7A,B). As the Cy5-ONs and diblock FITC-pEG-pEI molecules organize into multimolecular complexes, the number of free Cy5-ONs and free FITC-pEG-pEI strands in the focal volume is lowered, which is reflected in the FFS curves. Third, highly intense fluorescence peaks are observed in the two detectors at the same time. The simultaneous appearance of the red and green peaks indicates that a complex bearing many red-labeled as well as many green-labeled components is passing through the focal volume. It proves that the pEG-pEI/ONs complexes are indeed multimolecular. Others have also reported the multimolecular nature of this class of polyplexes. Vinogradov et al. determined the molecular weight (by static light scattering) of similar pEG-pEI/ONs complexes and calculated that they consist of 23 polymer strands and 24 oligonucleotides.[20] Harada et al. calculated for similar core−shell-type pEG-pLL/ONs complexes a composition of 162 (20-mer) or 248 (15-mer) ONs and hundreds of polymer chains.[21] Simultaneously appearing peaks of high fluorescence intensity could be observed for both 20-mer and 25-mer Cy5-ONs, when complexed to diblock FITC-pEG-pEI. Though gel electrophoresis showed that diblock FITC-pEG-pEI is not able to fully complex 20-mer Cy5-ONs (Figure 6, lanes 2−4), FFS showed that at least part of the Cy5-ONs are organized in multimolecular polyplexes.

Excessive addition of NaCl clearly influences the fluorescence fluctuation profiles (Figure 7E,F). The average background fluorescence intensity almost completely restores to the values of free Cy5-ONs (Figure 7A) and free FITC-pEG-pEI (Figure 7B). Moreover, highly intense fluorescence peaks completely disappear. Both phenomena indicate the dissociation of the diblock FITC-pEG-pEI/Cy5-ONs complexes. Lane 4 in Figure 9 indeed shows that adding NaCl to the diblock FITC-pEG-pEI/Cy5-ONs complexes prior to loading them on gel results in the release of a significant amount of Rh-ONs, which can migrate freely through the gel. Addition of dextran sulfate to the diblock FITC-pEG-pEI/Cy5-ONs complexes also resulted in the complete disappearance of highly intense fluorescence peaks in the FFS curves (data not shown). No threshold value could be calculated anymore in the fluorescence fluctuation profile of the labeled ONs, while autocorrelation analysis indeed was able to determine a diffusion coefficient equal to that of the free labeled ONs. Dextran sulfate is an anionic polymer, which competes with the Cy5-ONs for binding to the pEG-pEI chains and dissociates the diblock FITC-pEG-pEI/Cy5-ONs complexes as revealed from lane 5 in Figure 9.

When complexing Cy5-ONs (both 20-mer and 25-mer) with multiblock FITC-pEG-pEI, peaks of high fluorescence intensity were recorded simultaneously by both detectors (Figure 8C,D) and also the average background fluorescence intensity significantly decreased. Upon adding NaCl, this average background fluorescence intensity only partly restored (compare Figure 8E,F with respectively Figure 8A,B). Also, a few highly intense fluorescence peaks remained. It indicates that adding NaCl only partly dissociates the multiblock FITC-pEG-pEI/Cy5-ONs complexes. Indeed, lane 10 in

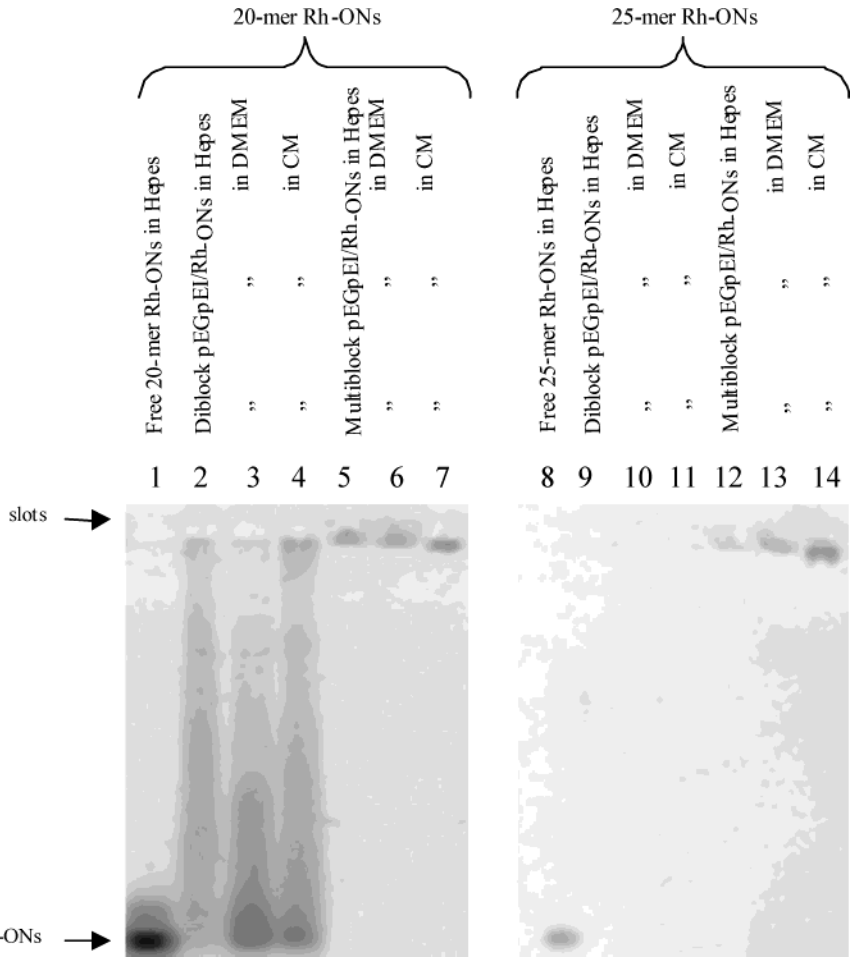

Figure 6. Gel electrophoresis on free Rh-ONs (lanes 1 and 8), diblock pEG-pEI/Rh-ONs complexes (lanes 2−4 and 9−11), and multiblock pEG-pEI/Rh-ONs complexes (lanes 5−7 and 12−14) in various media (Hepes buffer, Dulbecco's Modified Eagle Medium without serum (DMEM) and with serum (CM)). Lanes 1−7 show 20-mer Rh-ONs, and lanes 8−14 show 25-mer Rh-ONs. The Rh-ONs concentration in all the dispersions was 20 μg/mL; N/P equaled 15.

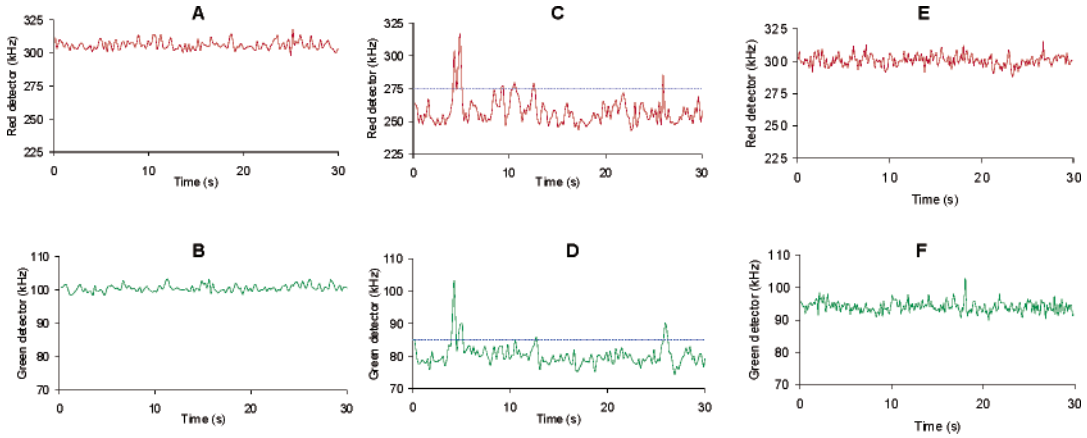

Figure 7. Fluorescence fluctuation profiles of a solution of free Cy5-ONs (A) and free diblock FITC-pEG-pEI (B) as registered by respectively the red and green detector. Fluorescence fluctuation profiles of a diblock FITC-pEG-pEI/Cy5-ONs dispersion (N/P = 15; 0.2 μg/mL 20-mer Cy5-ONs) as simultaneously registered by the FFS "red detector" (C) and "green detector" (D). Fluorescence fluctuation profiles of the diblock FITC-pEG-pEI/Cy5-ONs dispersion after addition of NaCl (final concentration 0.5 M), as simultaneously registered by the FFS "red detector" (E) and "green detector" (F). When applicable, threshold fluorescence intensity values are indicated as dotted lines, discriminating the highly intense fluorescence peaks (which occur as a multimolecular complex diffuses through the focal volume) from the average background fluorescence intensity.

Figure 9 shows that only a minor amount of the Rh-ONs is able to migrate freely through the gel. Addition of dextran sulfate to the multiblock FITC-pEG-pEI/Cy5-ONs complexes resulted in the complete disappearance of highly intense fluorescence peaks in the FFS curves (data not shown). Also, gel electrophoresis shows that dextran sulfate is able to completely release the ONs from the multiblock pEG-pEI/ONs complexes (lane 11 in Figure 9).

In the FFS experiments described above, the fluorescent marker (FITC) was attached to the pEG-strand of pEG-pEI, thus residing at the surface of the core−shell

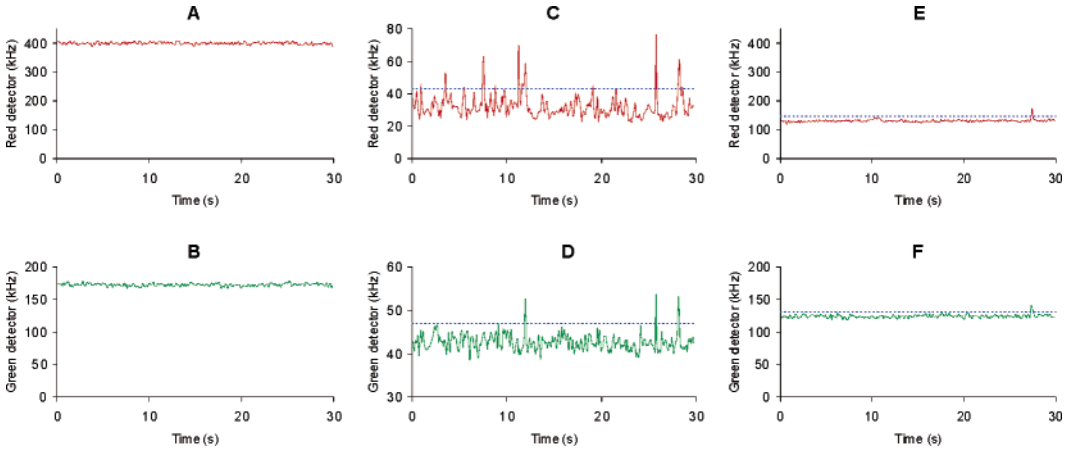

Figure 8. Fluorescence fluctuation profiles of a solution of free Cy5-ONs (A) and free multiblock FITC-pEG-pEI (B) as registered by respectively the red and green detector. Fluorescence fluctuation profiles of a multiblock FITC-pEG-pEI/Cy5-ONs dispersion (N/P = 15; 0.2 μg/mL 20-mer Cy5-ONs) as simultaneously registered by the FFS "red detector" (C) and "green detector" (D). Fluorescence fluctuation profiles of the multiblock FITC-pEG-pEI/Cy5-ONs dispersion after addition of NaCl (final concentration 0.5 M), as simultaneously registered by the FFS "red detector" (E) and "green detector" (F). When applicable, threshold fluorescence intensity values are indicated as dotted lines.

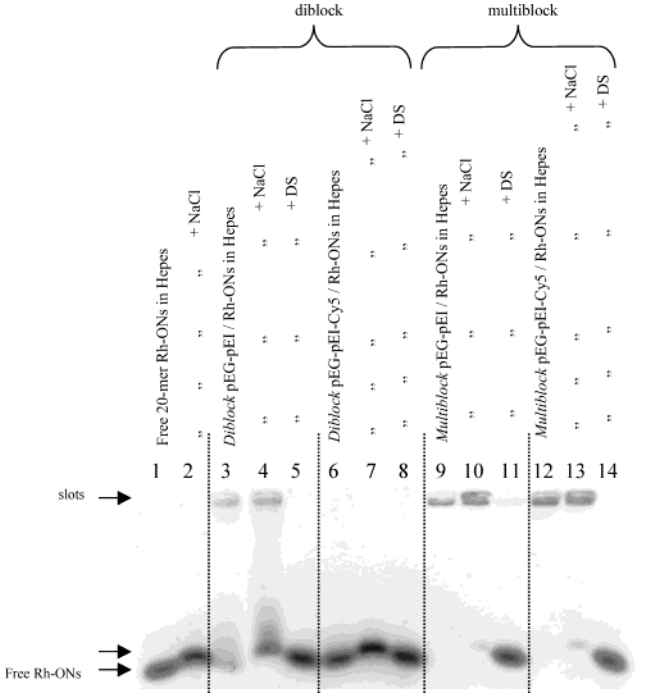

Figure 9. Gel electrophoresis on free 20-mer Rh-ONs (lanes 1 and 2), diblock pEG-pEI/Rh-ONs complexes (lanes 3−5), diblock pEG-pEI-Cy5/Rh-ONs complexes (lanes 6−8), multiblock pEG-pEI/Rh-ONs complexes (lanes 9−11), and multiblock pEG-pEI-Cy5/Rh-ONs complexes (lanes 12−14). The Rh-ONs concentration in all the dispersions was 20 μg/mL; N/P equaled 15. Addition of salt (NaCl) or dextran sulfate (DS) to the complexes (prior to loading them on gel) clearly destabilizes (i.e., dissociates) the complexes.

polyplexes. In the following experiments, the fluorescent label (Cy5) was bound to the cationic pEI segment of pEG-pEI, which takes part in the electrostatic interactions between the ONs and the polymer.

Dual color FFS measurements on the diblock pEG-pEI-Cy5/Rh-ONs dispersions did not reveal the simultaneous appearance of highly intense fluorescence peaks in the red and green detector or any significant changes in the average background fluorescence intensity of the FFS curves (data not shown), indicating that multimolecular complexes did not exist. Gel electrophoresis experiments indeed confirmed these results: as revealed from lane 6 in Figure 9, the diblock pEG-pEI-Cy5 was not able to complex the Rh-ONs anymore, in contrast with the unlabeled polymer (compare lane 6 with lane 3 in Figure 9). The fluorophores on the cationic segment of the diblock pEG-pEI clearly hinder the complexation of this polymer with Rh-ONs.

The gel electrophoresis experiments show that multiblock pEG-pEI-Cy5, like the nonlabeled copolymer, does complex with the Rh-ONs (compare lane 12 with lane 9 in Figure 9). Indeed, FFS measurements reveal a significant decrease of the average background fluorescence intensity, indicating that the amount of noncomplexed Rh-ONs and pEG-pEI-Cy5 molecules has decreased (compare Figure 10C,D with respectively Figure 10A,B). However, highly intense fluorescence peaks did not appear at all in the red and green fluorescence fluctuation profiles. A plausible explanation could be a strong quenching, as the Rh labels on the ONs and the Cy5 labels on the pEI segment of the polymer are in each other's proximity in the core of the polyplexes. On the contrary, for FITC-pEG-pEI/Cy5-ONs complexes, the FITC label of the polymer is located at the surface of the pEG shell, distinct from the Cy5-labeled ONs in the core of the complex. Apparently, the quenching effect overrules the increase in fluorescence intensity due to the high number of fluorophores in one complex. Upon addition of NaCl, the average background fluorescence intensity only partly restored (Figure 10E,F), indicating that only part of the Rh-ONs is released from the polyplexes. This could also be observed from gel electrophoresis measurements: Figure 9 (lanes 10 and 13) shows that both nonlabeled multiblock pEG-pEI and multiblock pEG-pEI-Cy5 remain able to complex a major part of the Rh-ONs after addition of NaCl.

Upon adding dextran sulfate to multiblock pEG-pEI/Rh-ONs complexes prior to loading them on gel, the Rh-ONs were completely released from the complexes as they migrate freely through the gel (lanes 11 and 14 in Figure 9). In FFS measurements, adding dextran sulfate to a dispersion of multiblock pEG-pEI-Cy5/Rh-ONs complexes indeed resulted in the recovery of the average background fluorescence intensity (data not shown).

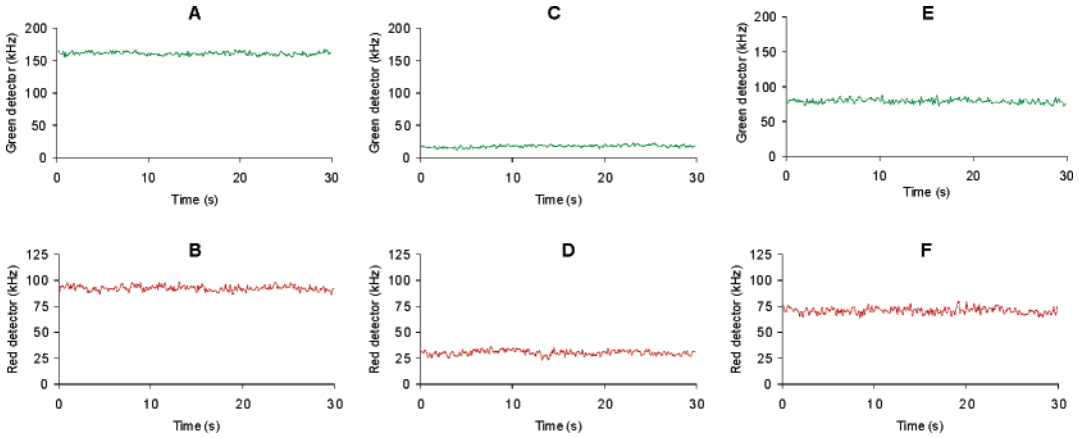

Figure 10. Fluorescence fluctuation profiles of a solution of free Rh-ONs (A) and free multiblock pEG-pEI-Cy5 (B) as registered by respectively the red and green detector. Fluorescence fluctuation profiles of a multiblock pEG-pEI-Cy5/Rh-ONs dispersion (N/P = 15; 0.2 μg/mL 20-mer Cy5-ONs) as simultaneously registered by the FFS "green detector" (C) and "red detector" (D). Fluorescence fluctuation profiles of the same multiblock pEG-pEI-Cy5/Rh−ONs dispersion after addition of NaCl (final concentration 0.5 M), as simultaneously registered by the FFS "green detector" (E) and "red detector" (F).

Conclusion and Summary

For both diblock and multiblock pEG-pEI, the complexation with oligonucleotides resulted in the formation of pegylated DNA complexes with a core−shell structure, as evidenced by ζ-potential measurements. However, differences in complexation behavior between the two types of pEG-pEI were observed. Gel electrophoresis demonstrated that the diblock pEG-pEI showed better complexation behavior for 25-mer Rh-ONs than for 20-mer Rh-ONs, whereas multiblock pEG-pEI was able to fully complex both types of Rh-ONs. In addition, conventional fluorimetry revealed that the fluorescence of Rh-labeled ONs was differently quenched upon complexation with diblock and multiblock pEG-pEI.

Dual color FFS measurements were performed on pEG-pEI/ONs complexes, the ONs and pEG-pEI chains being labeled with spectrally different fluorophores. In cases where the fluorescent label (FITC) was attached to the free end of the pEG segment of pEG-pEI, dual color FFS measurements on FITC-pEG-pEI/Cy5-ONs (for diblock as well as multiblock pEG-pEI) showed that both detectors simultaneously registered highly intense fluorescence signals. This indicates that a group of oligonucleotides and polymer chains migrates together through the detection volume and therefore proves that complexes are formed, which are composed of many oligonucleotide and polymer strands. These observations prove the formation of multimolecular polyplexes, which is in agreement with the gel electrophoresis results and observations in the literature. When the pEG-pEI polymers were fluorescent-labeled at their cationic pEI segment, however, FFS did not detect multimolecular polyplexes. In the case of diblock pEG-pEI-Cy5, the absence of peaks of high fluorescence intensity was indeed due to the fact that no multimolecular polyplexes could be formed, as was shown by gel electrophoresis. However, gel electrophoresis showed that multiblock pEG-pEI-Cy5 did complex Rh-ONs. The absence of highly intense fluorescence peaks in FFS was due to the mutual quenching of both the Cy5 and Rh labels that were in close proximity in the core of the core−shell complex. From these results, it could be concluded that dual color FFS allows studying the association and dissociation of pEG-pEI/ONs provided that the fluorescent label of the polymer is attached to the distal end of the pEG chain.

FFS is a very sensitive technique. Experiments can be done on small sample volumes in the nanomolar concentration range. Compare 50 μL of 0.2 μg/mL ONs for FFS measurements with 30 μL of 20 μg/mL ONs for gel electrophoresis and 1 mL of 10 μg/mL ONs for dynamic light scattering, ζ-potential, and conventional fluorimeter experiments. The very low concentrations used are a major advantage when studying scarce DNA and polymer material. The small sample volume required makes the technique suitable for measurements in rare media (e.g., cytosolic lysates) and in living cells. As a microscopy-based technique, FFS shows potential to study the biophysical behavior of DNA complexes in living cells. A major advantage of dual color FFS over single color FFS in studying the complexation behavior of DNA complexes in cells is that it might yield a considerable improvement in signal specificity. In heterogeneous media such as cells, the DNA and the cationic polymers may interact not only with each other but also with many other biological components. After dissociation of the DNA complex in the cytoplasm, aspecific binding of fluorescent DNA to cellular components could also result in peaks of high fluorescence intensity in the fluctuation profile. In single color FFS, this would lead to the false conclusion that the original DNA complexes have not dissociated. In dual color FFS, the absence of simultaneously occurring peaks in both detector channels would indicate that the original DNA complexes have already dissociated. Since gel electrophoresis can tell us whether Rh-ONs are free (migrating toward the anode) or complexed (remaining in the slots), so might dual color FFS be able to determine in the cytoplasm of a living cell whether the Rh-ONs are still complexed to their carrier or not. All this indicated by the presence or absence of simultaneous peaks in both the red and green detector channel. However, the sample material has to be analyzed with care: we have demonstrated the possibility of introducing artifacts as the fluorescent label may alter the physicochemical behavior of the polymer. Diblock pEG-pEI-Cy5 did not complex the Rh-ONs anymore, possibly due to steric hindrance of the fluorophore. Also, unforeseen quenching phenomena can result in the absence of dual color peaks while the Rh-ONs are complexed to their carrier (as observed for multiblock pEG-pEI-Cy5/Rh-ONs complexes).

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