Interactions Between Oligonucleotides and Cationic Polymers Investigated by Fluorescence Correlation Spectroscopy

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Purpose. To evaluate whether fluorescence correlation spectroscopy (FCS) can be used to characterize the complexation between oligonucleotides and cationic polymers.

Methods. The features of the complexes between rhodamine labeled oligonucleotides (Rh-ONs) and poly(2-dimethylamino)ethyl methacrylate (pDMAEMA), poly(ethylene glycol)-poly(ethyleneimine) (pEG-pEI), and diaminobutane-dendrimer-$(NH_2)_{64}$ (DAB_{64}) were characterized by light scattering, electrophoretic mobility, electrophoresis, and FCS.

Results. At low polymer/Rh-ON ratios, a decrease of the fluorescence of the Rh-ONs was observed on binding of the Rh-ONs to all cationic polymers. This was explained by the creation of "multimolecular complexes" in which the Rh-labels quench each other. The multimolecular complexes, which are highly fluorescent as they carry a number of Rh-ONs, resulted in high fluorescence peaks in the fluorescence fluctuation profile as measured by FCS. For pDMAEMA and DAB_{64}, at higher polymer/Rh-ON ratios the fluorescence of the polyplexes increased, caused by the formation of "monomolecular complexes," which consist of only one Rh-ON per polymer. In the case of pEG-pEI, the fluorescence stayed constant when the polymer/Rh-ON ratio increased, so multimolecular polyplexes remained. FCS confirmed these results as the high fluorescence peaks disappeared in case of pDMAEMA/Rh-ON and DAB_{64}/Rh-ON dispersions, but remained present for pEG-pEI/Rh-ON dispersions.

Conclusions. FCS seems applicable for study of the interactions between ONs and different types of cationic polymers.

KEY WORDS: dendrimers; FCS; multimolecular complexes; oligonucleotides; cationic polymers.

INTRODUCTION

In the last few years, antisense oligonucleotides (ONs) have become very attractive as a promising tool for the selective inhibition of gene expression and viral reproduction (1,2). The cellular uptake of ONs is poor due to their negatively charged backbone. In response to these problems, cationic lipids and cationic polymers, which spontaneously form complexes with the negatively charged nucleic acids, called lipoplexes and polyplexes, are under investigation as pharmaceutical carriers for ONs (3). At the moment the knowledge on self-assembly of cationic polymers and ONs is limited (4). However, from a standpoint of developing efficient DNA vectors, it is essential to be able to synthesize well-defined DNA complexes and characterize their physicochemical properties. Especially important is the understanding of their biological behavior.

A main biopharmaceutical step is the release of nucleic acid from the complex, which is essential in allowing interaction with their target. On the one hand, a strong affinity between the ONs and the cationic carriers might prevent the release of the ONs intracellularly. On the other hand, if the affinity is too low, the complex may dissociate in the blood or the extracellular environment. At the moment, many methods exist to study the complexation between DNA and cationic carriers in noncellular environments. To obtain real breakthroughs in the design and understanding of the dissociation of DNA complexes in a cellular environment, there is a need for physicochemical methods, which would allow us to characterize these critical steps not only in vitro but also in the cytoplasm of the cell where the dissociation has to occur.

With this purpose in mind we recently introduced fluorescence correlation spectroscopy (FCS) for studying the complexation of ONs to poly(2-dimethylamino)ethyl methacrylate (pDMAEMA; Fig. 1A), a cationic polymer under investigation as a DNA carrier for gene therapy (Fig. 1A) (5,6). FCS works on a microscopic scale and, consequently, shows potential to be applied in cells (7). As many types of cationic polymers are under investigation as carriers for DNA therapy, the present article aims to evaluate the extent to which FCS is applicable in the characterization of the complexation of DNA with cationic polymers. Polyplexes formed by three different types of cationic polymers were investigated (Fig. 1). First, complexes based on pDMAEMA, a linear polymer with positively charged side groups, were studied (8–10). Second, we studied core-shell polyplexes based on poly(ethylene glycol)-poly(ethyleneimine) (pEG-pEI; Fig. 1B), a block copolymer with a hydrophylic PEG segment (4). Several groups reported on the increased activity of ONs incorporated in such systems, both in vitro and well as in vivo (2). Third, we investigated the complexation between ONs and dendrimers that are uniform in size, show a highly branched spherical structure, and have a surface covered with charged primary amine groups (Fig. 1C) (10).

MATERIAL AND METHODS

Oligonucleotides

The rhodamine (Rh) labeled 25-mer phosphodiester ONs (5′-TCT-GGG-TCA-TCT-TTT-CAC-GGT-TGG-C-3′) (molar mass 8253 g/mol) was synthesized by Eurogentec (Seraing, Belgium). Each Rh-ON molecule contained one Rh label at the 5′ end of the ONs. The concentration of the Rh-ON stock solutions (in Tris-buffer at pH 8) was determined by absorption measurements at 260 nm (1 OD_{260} = 33 μg ON/ml). The contribution to the absorption at 260 nm by the Rh label was neglected in the determination of the concentration of the Rh-ONs.

Cationic Polymers

Poly(2-dimethylamino)ethyl methacrylate was synthesized at the University of Utrecht as described by van de

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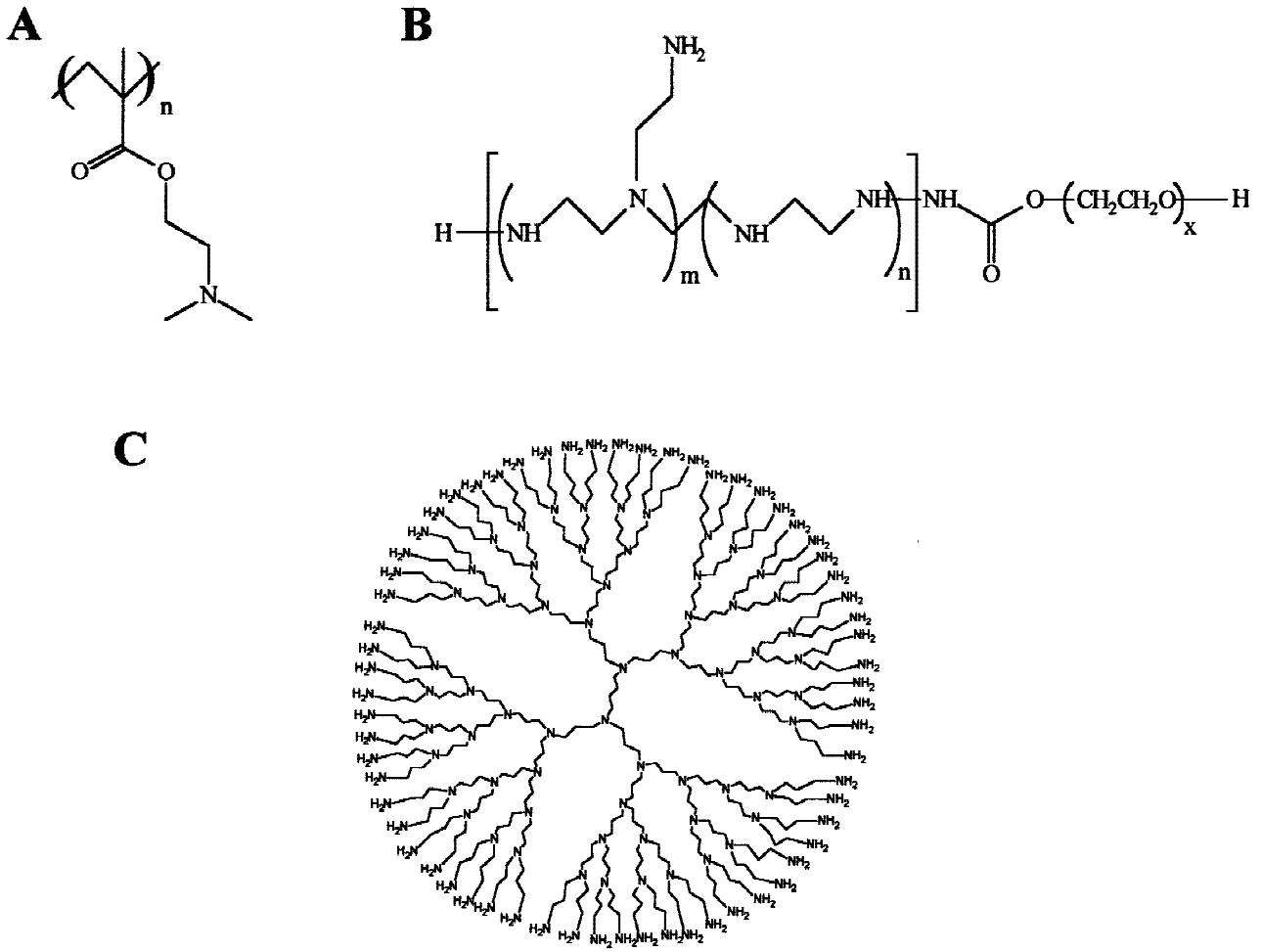

Fig. 1. Structure of the cationic polymers used: pDMAEMA (A), pEG-pEI (B), and DAB_{64} (C).

Wetering *et al.* (8). The molar mass of the DMAEMA monomer equals 157 g/mol. The number average molar mass (M_n) of pDMAEMA was estimated to be around 60,000 g/mol as determined by gel permeation chromatography. pDMAEMA contains tertiary amino groups with an average pKa of 7.5 (11).

Poly(ethylene glycol)-polyethyleneimine was synthesized at the University of Nebraska Medical Center as described by Vinogradov *et al.* (4). The total content of nitrogen was 3.75 μmol/mg polymer. The weight average molecular weight M_w was determined to be 12,400 g/mol by static light scattering.

DAB-dendrimer-$(NH_2)_{64}$ (DAB_{64}) was synthesized at the Eindhoven University by published methods (12,13). This type of dendrimer has 1,4-diaminobutane (DAB) as the central core molecule and bears 64 surface charges (amine groups). The molar mass is 7168 g/mol (13).

Preparation of Polyplexes

Complexes with different w/w (weight of the cationic polymer/weight of the Rh-ON) ratios were prepared: a w/w value of 1 indicates that an equal mass of cationic polymer and Rh-ON was present in the polyplex dispersion. The Rh-ON concentration in the polyplex dispersion was always 10 μg/ml (1.2 μM). The complexes (varying in w/w ratio) were prepared by adding in one step, different volumes of the cationic polymer stock solution (in HEPES buffer at pH 7.4) to a fixed volume of the Rh-ON stock solution. The polymer solutions were filtered before being added to the nucleotide solutions to avoid dust particles. Average pore size of the filter was 0.45 μm (Schleicher & Schuell, Dassel, Germany). After addition of the polymer solution, the dispersion was vortexed for 10 s. To obtain the final Rh-ON concentration of 10 μg/ml, the dispersions were further diluted with 20 mM HEPES buffer at pH 7.4. The polyplexes were allowed to equilibrate for 30 min at room temperature before use. Particle size, zeta potential, and fluorescence measurements were all done on the same cationic polymer/Rh-ON dispersions.

For FCS measurements, the cationic polymer/Rh-ONs dispersions were prepared as described above. However, the final Rh-ON concentration equaled 0.2 μg/ml (24 nM).

Particle Size Measurements

Thirty minutes after the preparation of the polyplexes, the particle size of the polyplexes was determined by dynamic light scattering measurements (DLS) at 25°C and at an angle of 90° with a Malvern 4700 instrument (Malvern, Worcestershire, U.K.) using a HeNe laser beam (633 nm). The polyplexes were prepared as described above. For the data analysis, the viscosity and refractive index of water at 25°C (0.89 mPa.s and 1.333, respectively) were used. Polystyrene nano-

spheres (220 ± 6 nm; Duke Scientific Corp., Palo Alto, CA) were used to check the performance of the instrument. The particle size of each dispersion was measured three times.

Zeta Potential Measurements

Within 1 h after preparation of the complexes, the zeta potential (ζ) was measured at a temperature of 25° with a Malvern Zetasizer 2000 (Malvern, Worcestershire, UK), which is based on electrophoretic light scattering. Polystyrene nanospheres (−50 mV; Duke Scientific Corp., Palo Alto, CA) were used to check the performance of the instrument. ζ of each dispersion was measured three times.

Fluorescence Measurements

Thirty minutes after their preparation, the fluorescence of cationic polymer/Rh-ON complexes (λ_{ex} = 525 nm, λ_{em} = 584 nm) was measured on an SLM-Aminco Bowman spectrofluorimeter (SLM-Aminco Bowman, Rochester, NY). The fluorescence of each dispersion was measured three times.

Agarose Gel Electrophoresis on the Polyplexes

The polyplex dispersions used in the gel electrophoresis experiments were prepared as already described but contained 26.7 μg/ml Rh-ON (3.2 μM). After their preparation the polyplexes were allowed to equilibrate for 30 min at room temperature. Thirty microliters of these dispersions were 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 (pH 8) 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 were detected based on their fluorescence.

Exchange of Rh-ON in Polyplexes with Dextran Sulfate as Monitored by Gel Electrophoresis

pDMAEMA/Rh-ON, pEG-pEI/Rh-ON, and DAB_{64}/Rh-ON polyplex dispersions were prepared, all having a w/w ratio of 4 and containing 26.7 μg/ml Rh-ON. Thirty minutes after their preparation, dextran sulfate, at various concentrations (10, 50, 100, 150, 200, 250, 300, 400, and 500 μg/ml) was added to the cationic polymer/Rh-ON complexes. Another 30 min later, 30 μl of these mixtures was diluted with 5 μl of a 50% sucrose solution in distilled water and placed in the wells of a 1.1% agarose gel to start the electrophoresis experiments, as described.

Fluorescence Correlation Spectroscopy

A commercial FCS setup (Confocor I, Zeiss-Evotec, Jena, Germany) was used. Basically, as illustrated in Fig. 2, FCS monitors the fluorescence fluctuations (Fig. 2B) in a femtoliter excitation volume, which is approximated as a cylinder with radius ω_1 and height $2\omega_2$ (Fig. 2A). On diffusion of a fluorescent labeled molecule through the excitation volume, the fluorophore emits photons that are detected by a highly sensitive avalanche photodiode. Fluorescence fluctuations can be caused by Brownian diffusion and flow and chemical reactions. In case the fluorescence fluctuations only result from the diffusion through the excitation volume, the autocorrelation function $G(\tau)$ (Fig. 2C) as a function of variable τ takes the following form (14):

$$G(\tau) = 1 + \frac{1}{N} f(\tau/\tau_d) \tag{1}$$

with

$$f(\tau/\tau_d) = \left[\frac{1}{1+\tau/\tau_d}\right]\left[\frac{1}{1+(\omega_1/\omega_2)^2\,\tau/\tau_d}\right]^{1/2} \tag{2}$$

The autocorrelation function $G(\tau)$ allows the determination of the translational diffusion time (τ_d), which characterizes the average residence time in the excitation volume and the average number of particles in the excitation volume (N).

The translational diffusion coefficient (D) can be calculated from τ_d by the following equation:

$$D = \frac{\omega_1^2}{4\tau_d} \tag{3}$$

As small molecules move more rapidly through the excitation volume than larger ones, a significant retardation of the diffusion of small fluorescent molecules can be expected when they interact with larger nonlabeled macromolecules. Due to the presence of molecules with a fast (τ_{free}) and slow (τ_{bound}) translational diffusion time, the autocorrelation function can be split into a fast and slow decay with the amplitude fractions representing the relative amount of free (1–y) and bound (y) ligand in the solution (14,15):

$$G(\tau) = 1 + \frac{1}{N}\left[\frac{(1-y)\cdot f(\tau/\tau_{free}) + \alpha^2\, y \cdot f(\tau/\tau_{bound})}{[(1-y)+y\,\alpha]^2}\right] \tag{4}$$

In case the free and bound ligand do not show the same fluorescence signal per molecule (fpm), we must invoke a correction factor α (fpm bound/fpm free) to prevent the fraction of the brightest particle from being overestimated in the autocorrelation function amplitude.

The FCS experiments were performed as follows. The light of a HeNe laser (543 nm) was projected into the microscope water immersion objective (C-apochromat 40 × 1.2 W) via a dichroic mirror. The laser power was attenuated by use of a neutral density filter. The laser beam was focused at about 180 μm above the bottom of the cuvettes, which contained the Rh-ON solutions or polyplex dispersions. The emitted light was collected by the same objective and passed through the dichroic mirror and the 45 μm pinhole to finally reach the avalanche photodiode. For FCS measurements on the cationic polymer/Rh-ON complexes, 200 μl of the dispersions were prepared as previously described in an Eppendorf tube and immediately transferred into the FCS Nunc cuvettes to begin the FCS measurement. In each FCS experiment the fluorescence fluctuations were measured for 50 s.

RESULTS AND DISCUSSION

Complex Formation as Revealed by Dynamic Light Scattering and Zeta Potential

Figure 3 shows the results of size and ζ measurements on the different types of polyplexes as a function of their w/w ratio. For pDMAEMA/Rh-ON dispersions, at low values of

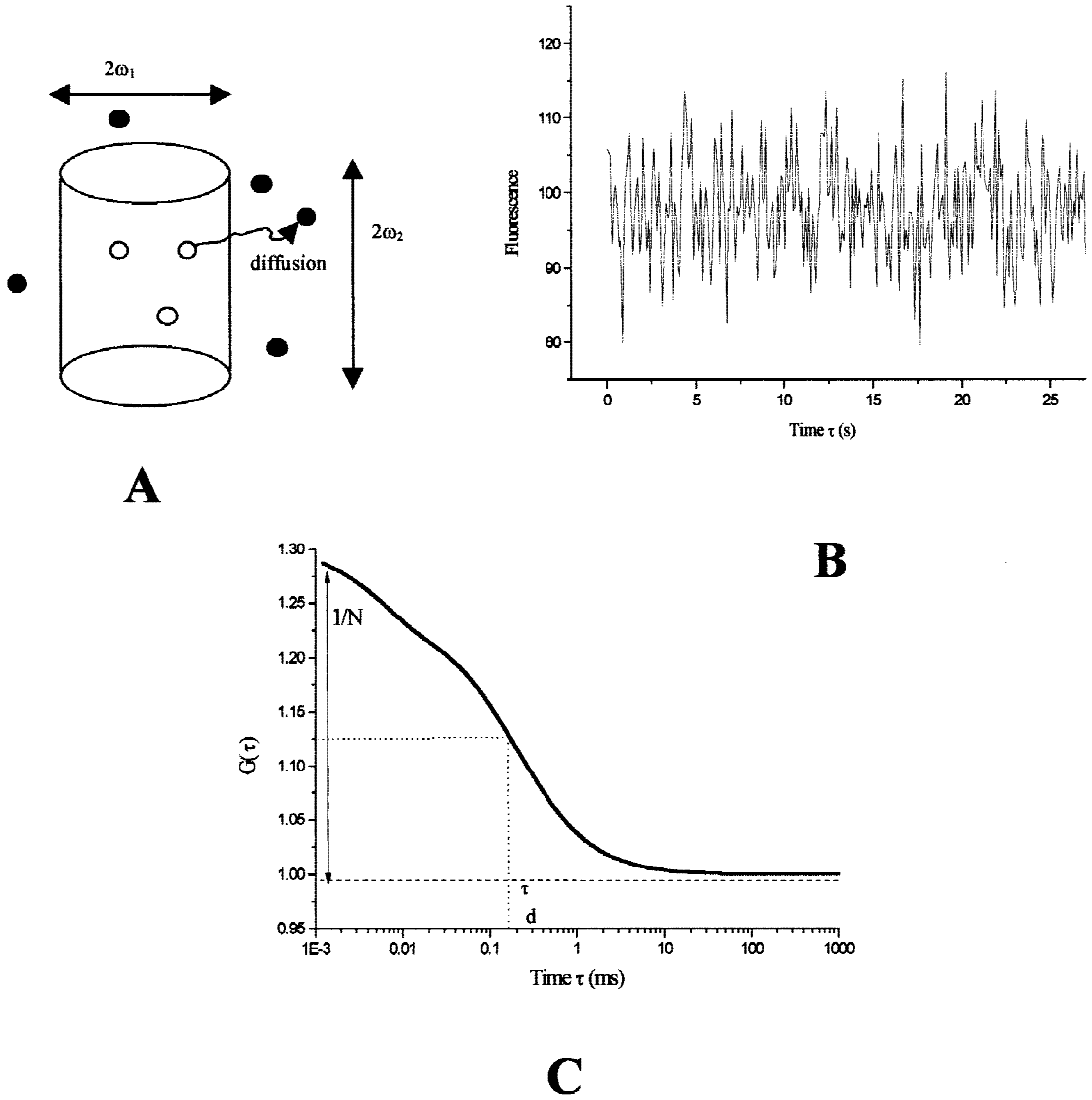

Fig. 2. The passage of fluorescent molecules through the excitation volume (A) gives rise to fluctuations in measured fluorescence (B). An autocorrelation function (C) can be derived from the fluctuations and allows calculating the average diffusion time τ_d and the average number of fluorescent molecules in the excitation volume (N).

w/w, the size of the polyplexes gradually increases with the increasing amount of pDMAEMA (6). At w/w of 2, aggregation of the pDMAEMA/Rh-ON polyplexes occurs, which is attributed to the almost neutral ζ of the polyplexes. Further increasing the amount of pDMAEMA results in a positive ζ, which probably indicates that a charged polycation "corona" surrounds the complexes. The positive charge of the pDMAEMA/Rh-ON polyplexes seems to prevent aggregation (Fig. 3A). Electrophoresis data proved that at high values of w/w all the Rh-ONs were bound (data not shown).

In the case of pEG-pEI, the size of the complexes hardly changes when the w/w ratio is varied. Although at low w/w values ζ sharply increases, at higher w/w ratios ζ levels off and remains neutral. It indicates that the excess pEG-pEI does not incorporate into the electroneutral particles, in contrast to the pDMAEMA/Rh-ON polyplexes at high w/w value. This (nearly) neutral value of ζ is consistent with the formation of a core-shell structure in which a PEG corona surrounds the ONs complexed to the pEI segments (16,17). The PEG corona prevents aggregation, as clearly observed from the DLS data (Fig. 3A).

The addition of DAB_{64} to the Rh-ON solutions results in large aggregates, comparable with the complexation of pDMAEMA to Rh-ONs. A main difference with pDMAEMA is that only at extremely high amounts of DAB_{64} (w/w > 15) a decrease in the particle size can be observed, although positively charged DAB_{64}/Rh-ON polyplexes already exist at w/w around 5 (Fig. 3B). Large aggregates from complexation of dendrimers with plasmid DNA were also reported by Kukowska-Latallo (18).

Complex Formation as Revealed by Fluorescence Measurements

Figure 4 shows that at lower w/w values the fluorescence of the Rh-ON solution decreases on complexation with all types of cationic polymers studied. Although the maximal

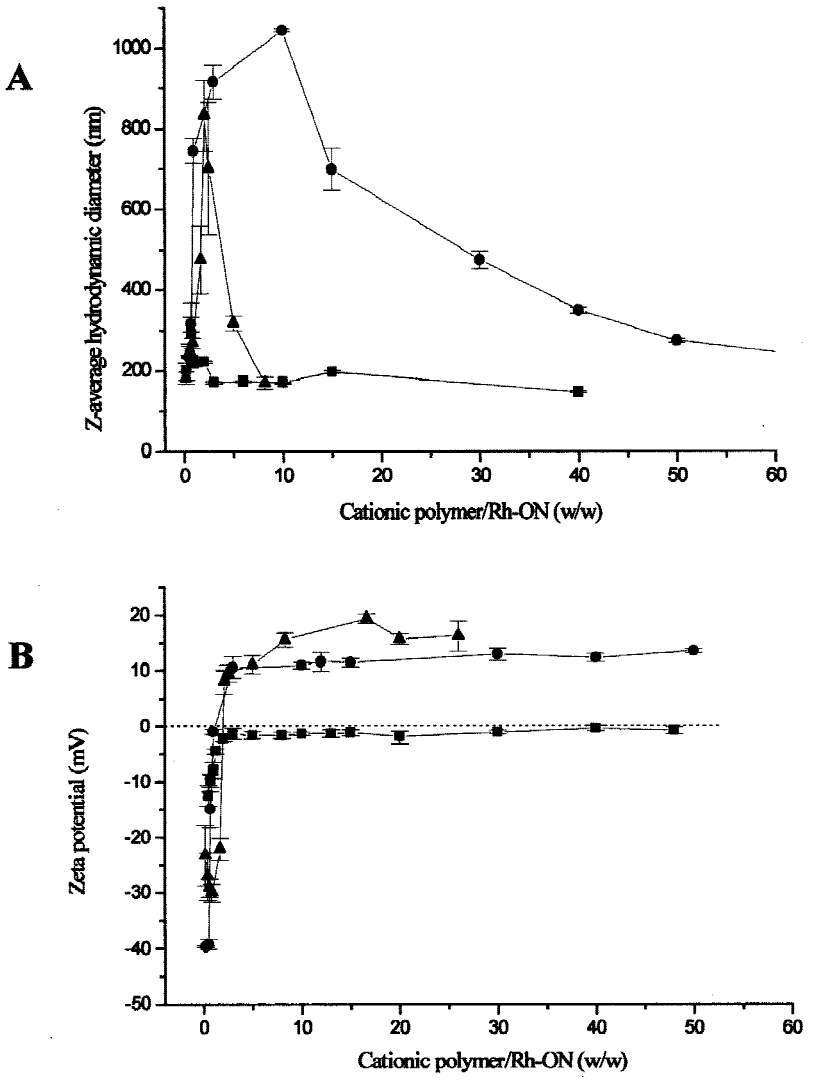

Fig. 3. Z-average hydrodynamic diameter (A) and zeta potential (B) of pDMAEMA/Rh-ON (▲), pEG-pEI/Rh-ON (■), and DAB_{64}/Rh-ON (●) dispersions as a function of the w/w ratio. The Rh-ON concentration in all the dispersions was 10 μg/ml ($n = 3$).

degree of quenching seems to be independent of the type of cationic polymer, there was a clear difference in the degree of quenching at higher w/w values.

In a previous study we showed experimental evidence that the decrease in the fluorescence of Rh-ONs on complexation with pDMAEMA is attributed to partial quenching of the fluorescence of the Rh-label due to the presence of several Rh-ON chains in one complex (*i.e.*, "multimolecular complexes") (6). Thereby, the labels are in close proximity and quench each other. The increased fluorescence of the pDMAEMA/Rh-ON polyplexes at higher w/w was explained by a decrease in the number of Rh-ON per polyplex; at high w/w values, a higher number of polyplexes but with a lower number of Rh-ONs per polyplex were present, resulting in less quenching between the Rh labels. Also, when pDMAEMA/Rh-ON polyplexes with high w/w were prepared by titrating a pDMAEMA/Rh-ON dispersion having low w/w with a pDMAEMA solution (instead of adding the pDMAEMA solution in one step to the Rh-ON stock solution as in Fig. 4), the fluorescence increased at high w/w values. This indicated that a "redistribution" of the Rh-ONs took place; Rh-ONs originally present in a specific complex were released from this complex to become attached to other cationic polymers. The reason the fluorescence at high w/w values largely exceeds the fluorescence of the free Rh-ON solution is unclear but could be attributed to the presence of the pDMAEMA chains, which probably results in another surrounding of the Rh-ONs, which may alter their fluorescence properties.

In the case of pEG-pEI, at high w/w ratios the fluorescence stayed constant, indicating that the structure of the pEG-pEI/Rh-ON complexes at high w/w ratios seems similar to the one at lower w/w ratios. This is supported by Fig. 3; size and ζ of the complexes hardly changed at higher w/w ratios. Also, when the Rh-ON solutions were titrated with pEG-pEI solutions, the fluorescence remained constant, indicating that a "redistribution" of the Rh-ON chains did not occur. It seems that the Rh-ON chains are entrapped in the core of the

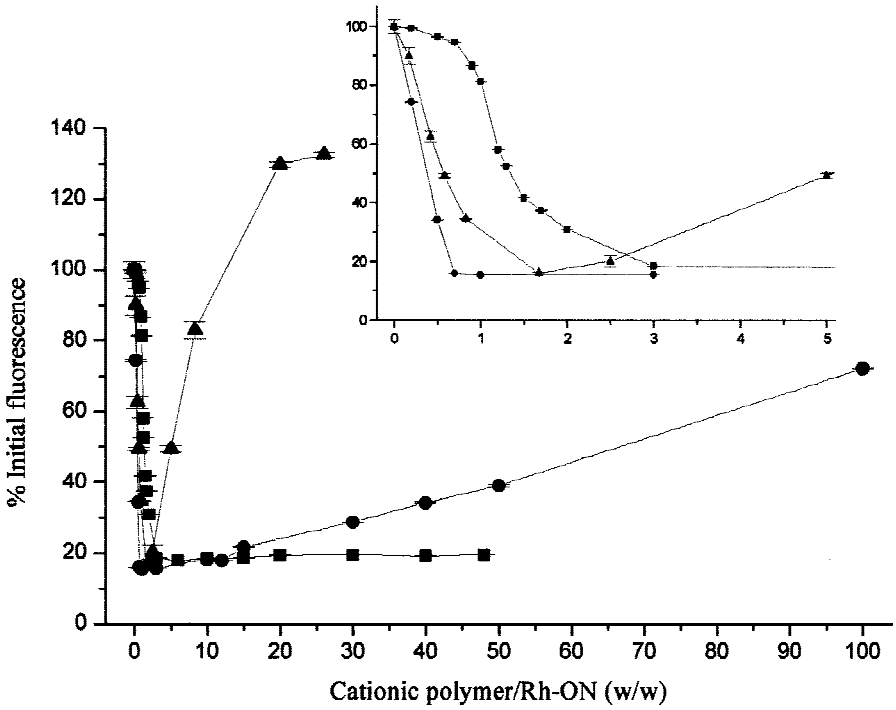

Fig. 4. Fluorescence (as measured by a fluorimeter) of pDMAEMA/Rh-ON (▲), pEG-pEI/Rh-ON (■), and DAB_{64}/Rh-ON (●) dispersions as a function of the w/w ratio. The Rh-ON concentration in all the dispersions was 10 μg/ml: consequently, the number of Rh-ON chains was the same in all the dispersions. The fluorescence of the Rh-ON solution in the absence of cationic polymer (*i.e.*, at w/w = 0) was set to 100% (n = 3).

complexes and are not able to leave the complex to become attached to other pEG-pEI strands.

As for the pDMAEMA/Rh-ON polyplexes, the fluorescence of the DAB_{64}/Rh-ON polyplexes increased at higher w/w values; however, only from w/w value >15 upward.

Complex Formation as Revealed by Agarose Gel Electrophoresis

Results of agarose gel electrophoresis experiments on pDMAEMA/Rh-ON polyplexes showed that when the amount of pDMAEMA was increased, the amount of free Rh-ON was diminished, whereas the amount of complexed Rh-ON (which remains in the slots) increased (data not shown). From a w/w value of 2 upward, all the Rh-ON chains were associated with the pDMAEMA chains. This is exactly the w/w ratio at which ζ approaches zero (Fig. 3B), whereas the fluorescence of the pDMAEMA/Rh-ON polyplexes was minimal at this w/w value (Fig. 4). Similarly, in the case of pEG-pEI, the amount of free Rh-ON also decreased when more pEG-pEI was added, whereas no free Rh-ON could be

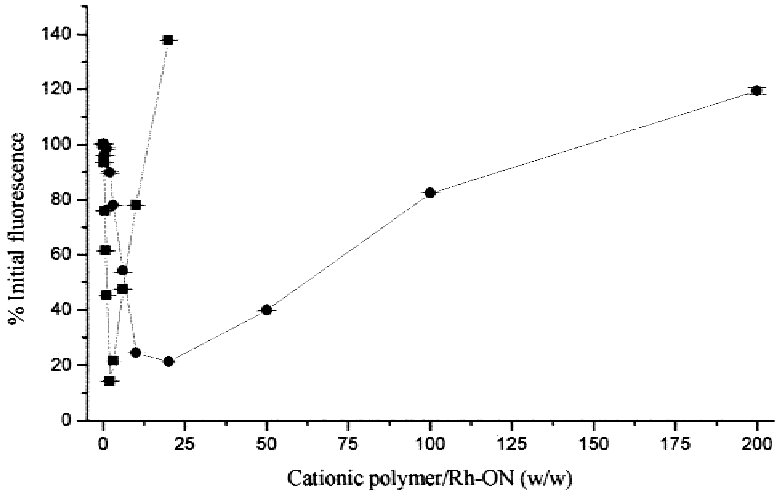

Fig. 5. Fluorescence (as measured by a fluorimeter) of pDMAEMA/Rh-ON dispersions. The Rh-ON concentration in all the dispersions was 10 μg/ml (■) and 0.1 μg/ml (●), respectively. The fluorescence of the Rh-ON solution in the absence of cationic polymer (*i.e.*, at w/w = 0) was set to 100% (n = 3).

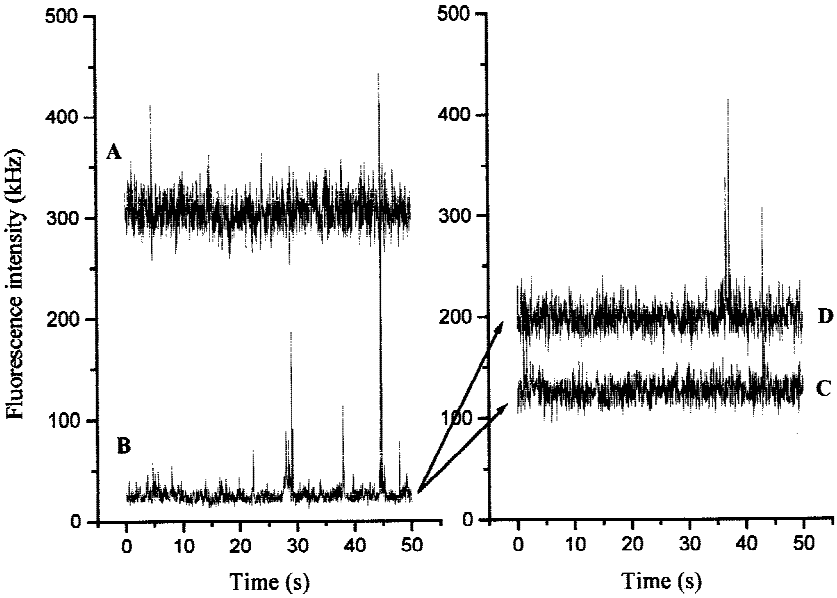

Fig. 6. Left panel: fluorescence fluctuations in the excitation volume of the FCS instrument for a Rh-ON solution (A) and pDMAEMA/Rh-ON dispersions (B: w/w = 10). The Rh-ON concentration equaled 0.2 μg/ml (24 nM). Right panel: adding 3 μg/ml dextran sulfate (C) to the pDMAEMA/Rh-ON dispersions partially recovered the fluorescence of the baseline. The addition of 30 μg/ml (D) dextran sulfate resulted in a partial recovery of the fluorescence and a disappearance of the highly intense fluorescence peaks.

detected from a w/w value of 2 upward (data not shown). In the case of DAB_{64}, all the Rh-ONs were already associated to the cationic polymer at a w/w value of 0.5. The insert in Fig. 4 shows that the maximal degree of fluorescence quenching, which can be expected to occur when all the Rh-ONs are bound, was also obtained at a w/w value around 0.5. The reason complete binding of Rh-ONs to DAB_{64} occurs at a lower w/w value probably can be attributed to the higher charge density of DAB_{64} compared with pDMAEMA and pEG-pEI.

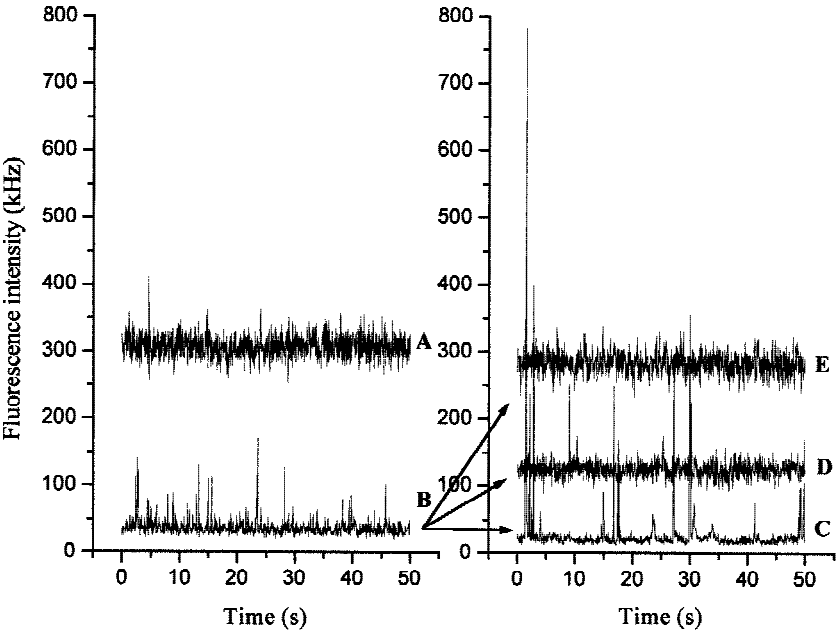

Fig. 7. Left panel: fluorescence fluctuation profile for a Rh-ON solution (A) and DAB_{64}/Rh-ON dispersions, respectively (B: w/w = 10). The Rh-ON concentration equaled 0.2 μg/ml (24 nM). Right panel: adding 3 μg/ml dextran sulfate (C) to the DAB_{64}/Rh-ON dispersions did not influence the fluorescence fluctuation profile of the polyplex. The addition of 12 μg/ml (D) and 30 μg/ml dextran sulfate (E) results, respectively, in a partial and total recovery of the fluorescence and an almost complete disappearance of the highly intense fluorescence peaks.

Complex Formation as Revealed by FCS

We became interested in studying interactions between Rh-ONs and cationic carriers by FCS because this method shows opportunities for intracellular measurements (7,19). Furthermore, we expected that FCS would be feasible to study ON-cationic polymer interactions, as on binding of (fluorescently labeled) ONs to cationic polymers their diffusion should slow down. This should result in a decreased diffusion coefficient as obtained by autocorrelation analysis of the fluorescence fluctuations.

The "A-data" in Figs. 6 and 7 are the fluorescence fluctuations in the excitation volume of free Rh-ONs (24 nM in HEPES buffer pH 7.4). From the mean diffusion time of the molecules (193 ± 4 μs), as calculated from autocorrelation analysis of the data, a diffusion coefficient of 1.05×10^{-6} cm^2/s was calculated (6). This agrees with values in the literature (20). The association of the Rh-ONs with the cationic polymers (pDMAEMA and DAB_{64}, respectively) clearly influenced the fluctuation profiles (B series in Fig. 6 and 7). Similar observations were made in case of pEG-pEI (data not shown). For all the polymers studied, the fluorescence intensity in the excitation volume significantly decreased, which agreed with the fluorimetric measurements (Fig. 4). Moreover, highly intense fluorescence peaks appeared in the fluorescence fluctuations, which were assumed to derive from the diffusion of "multimolecular" complexes (*i.e.*, complexes bearing numerous ON molecules, each ON molecule being labeled with a fluorescent tag) into the excitation volume. The diffusion coefficient as obtained by autocorrelation analysis of the fluorescence fluctuations between highly fluorescence peaks (further indicated as "baseline") allowed us to conclude that these fluctuations originated from the diffusion of the free Rh-ONs (data not shown). This seems contradictory to the gel electrophoresis results, which showed that at higher w/w values, all the Rh-ONs are bound. However, as FCS can detect single fluorescent molecules it is much more sensitive than the fluorescence detection used in gel electrophoresis. Moreover, there exists a major difference between the concentration used in FCS (nM) and electrophoresis experiments (μM), respectively. As illustrated in Fig. 5, diluting the dispersions may dissociate the polyplexes, which increases the concentration of free Rh-ONs.

Unfortunately, the highly fluorescent peaks drastically disturbed the analysis of the fluctuation profiles by autocorrelation and, consequently, binding studies by means of autocorrelation analysis was no longer feasible. To obtain information on the state of complexation of the Rh-ONs one has to rely on the presence or absence of highly fluorescent peaks in the fluctuation profile. To fully confirm that the highly fluorescent peaks are caused by complexed Rh-ONs, we further investigated (1) the average fluorescence of the (highly fluorescent) peaks of complexes that were prepared from cationic polymers and mixtures of nonlabeled and rhodamine labeled ON, (2) the fluorescence fluctuation profile at high w/w values, and (3) the change in the fluctuation profiles when dextran sulfate was added to the polyplex dispersions, which (partially or fully) dissociated the polyplexes.

Figure 8 shows the average fluorescence per peak as a function of the composition of the ON + Rh-ON mixture used in the preparation of pDMAEMA based polyplexes; although the total ON concentration (0.2 μg/ml) was fixed, only the ratio of Rh-labeled to unlabeled ON was varied. Three types of pDMAEMA polyplexes were prepared differing in w/w ratio. To identify whether a certain fluorescence value could be considered a highly fluorescent peak (*i.e.*, to find out whether it differed significantly from the intensity of the baseline) and to calculate the average fluorescence of the peaks (correcting for the intensity of the baseline attributed to free Rh-ON), we used a method described recently by Van Craenenbroeck *et al.* (21).

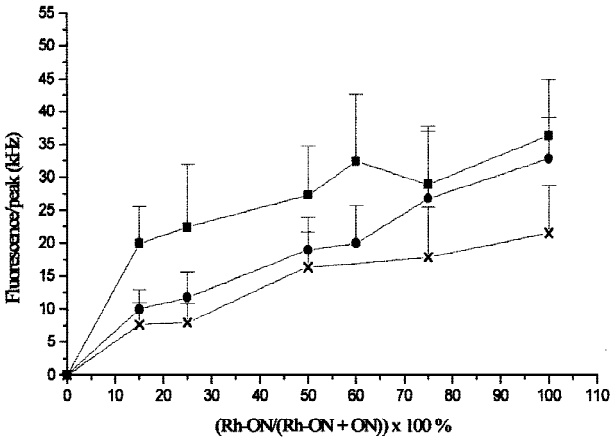

Fig. 8. The average fluorescence/peak of pDMAEMA/Rh-ON dispersions at different w/w ratios (w/w = 1, (■), w/w = 5 (●), and w/w = 20, (x)) as measured by FCS. The total ON concentration was fixed (0.2 μg/ml), the ratio of Rh-labeled to unlabeled ON was varied. The x-axis indicates the percentage of Rh-ON in the "ON + Rh-ON mixtures."

Three observations were made from Fig. 8. First, large standard deviations were present, which is due to the heterogeneous composition of the complexes (*e.g.*, with regard to the number of Rh-ONs per complex) that results in a broad distribution of the height of the highly intense fluorescent peaks. Second, in all w/w values, the higher the amount of labeled ONs in the ON + Rh-ON mixture, the higher the average fluorescence of the peaks. In confirms that the highly intense fluorescence peaks derive from multimolecular complexes that are composed of a number of Rh-ONs. However, pDMAEMA-based polyplexes prepared from an ON + Rh-ON mixture containing a low percentage of Rh-ON (*e.g.*, 15%) were not always significantly less fluorescent than polyplexes prepared from only Rh-ONs (*i.e.*, 100% Rh-ON). This can be attributed to the quenching of the fluorescence, which becomes especially pronounced when many Rh-ONs are present in the complex. Third, in all compositions of the ON + Rh-ON mixture, the higher the w/w ratio, the lower the average fluorescence per peak, indicating that the individual polyplexes become less fluorescent. This agrees with the fluorescence (as measured by a fluorimeter) of the 0.1 μg/ml (*i.e.*, 12 nM) polyplex dispersions as shown in Fig. 5.

At high w/w ratios highly intense fluorescence peaks in the fluctuations were no longer observed in the case of Rh-ON/pDMAEMA and Rh-ON/DAB_{64} (data not shown). As previously discussed, the increase in the fluorescence at higher w/w values as observed in Fig. 4 was explained by less quenching between the Rh labels due to a lower number of Rh-ONs in the complexes. The existence of "monomolecular" complexes, consisting of only one or a few Rh-ONs, probably explains why highly fluorescent peaks were not observed

at high w/w values. Contrary to pDMAEMA and DAB_{64}, highly intense fluorescence peaks remained present in the fluorescence fluctuations of Rh-ON/pEG-pEI dispersions at high w/w ratios (data not shown). Again, this agrees with the observations in Fig. 4 indicating that multimolecular Rh-ON/pEG-pEI complexes continue to exist at higher w/w ratios.

Another indication that the highly intense fluorescence peaks are related with complexation of the ONs was obtained from dissociation of the polyplexes by the poly-anion dextran sulfate. In particular, characterization of the dissociation of DNA complexes in cells is a question of principal importance with regard to the use of cationic polymers as DNA carriers. When dextran sulfate was mixed with the polyplex dispersions, two features in the fluorescence fluctuation profiles indicated the release of Rh-ONs from the multimolecular complexes (compare profiles C, D, and E vs. B in Figs. 6 and 7). First, the intensity of the baseline increased, which indicates a higher amount of free Rh-ONs. Second, the highly intense fluorescence peaks (partially or fully) disappeared. In the case of pDMAEMA (Fig. 6), the addition of dextran sulfate (3 or 30 μg/ml) could only partially release the Rh-ONs from the complexes. This agreed with the results from electrophoresis measurements that also indicated even a very high concentration (500 μg/ml) of dextran sulfate was not able to release all the ONs from the pDMAEMA/Rh-ON complexes. In the case of DAB_{64} (Fig. 7) the addition of 3 μg/ml of dextran sulfate did not influence the fluorescent fluctuations; 30 μg/ml was necessary to have a complete disappearance of the multimolecular complexes. In the case of pEG-pEI, 3 μg/ml of dextran sulfate resulted in the dissociation of all the multimolecular complexes (data not shown).

Finally, FCS seems applicable to study interactions between fluorescently labeled ONs and cationic polymers, independent of the type of cationic polymer. As FCS measurements can be done in cells, it may become a tool to investigate the complexation between DNA and cationic carriers in cells, which is a crucial step in the optimization of pharmaceutical carriers for DNA.

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