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Fluorescence Fluctuation Analysis for the Study of Interactions between Oligonucleotides and Polycationic Polymers

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The interactions between a cationic polymer, poly(2-dimethylamino)ethyl methacrylate (pDMAEMA), and negatively charged rhodamine-labeled 25-mer phosphodiester oligonucleotides (Rh-ONs) were studied by fluorescence fluctuation spectroscopy and other techniques. The composition of the pDMAEMA/Rh-ON complexes was investigated as a function of the charge ratio (+/-) by increasing the pDMAEMA concentration and keeping the Rh-ON concentration constant. We applied two different methods for analyzing the fluorescence fluctuation profiles of the pDMAEMA/Rh-ON complexes, which depended on their composition. First, we analyzed the data with the photon counting histogram (PCH) technique, which determines the molecular brightness and the concentration of fluorophores (Chen *et al.*, 1999). A particular challenge for the data analysis is the occurrence of sudden fluorescence bursts in the fluorescence fluctuation profiles, which are linked to the appearance of multimolecular complexes (*i. e.* when several Rh-ONs were present in one complex). A quantitative interpretation of the analysis for the complexes remains challenging and is connected to the rarity of the fluorescent bursts, which do not provide sufficient data statistics. To specifically address the problem of the fluorescent bursts we employed a method described by Van Craenenbroeck *et al.* (1999). This method, applicable only when data were integrated over much longer time bins, allowed us to estimate the number of fluorescence bursts which could be considered as a relative measure of the amount of multimolecular complexes present. When monomolecular complexes were formed, *i. e.* at high

values of the charge ratio, highly intense fluorescence peaks were not present and the interpretation of the PCH analysis was more straightforward. The molecular brightness of the species (ϵ), as revealed from PCH analysis, was greater than ϵ for the free Rh-ONs, indicating that the Rh-ONs were attached to pDMAEMA chains.

Key words: Fluorescence correlation spectroscopy/ Gene therapy/Interpolyelectrolyte complexes/Photon counting histograms/Polyplexes/Self-assembling systems.

Introduction

For the downregulation of specific gene products, entry of oligonucleotides (ONs) into the cytoplasm or nucleus is necessary to obtain interaction with its specific target (Cohen *et al.*, 1994). Due to their large size, hydrophilicity and negative charge the cellular uptake of ONs is very low. Therefore, to bypass these problems, cationic polymers which spontaneously form interpolyelectrolyte complexes with ONs ('called polyplexes') are under investigation as pharmaceutical carriers (De Smedt *et al.*, 2000). To obtain breakthroughs in the design and the understanding of the dissociation of polyplexes in cells, there is a need for advanced physicochemical methods, which allow the characterization of these critical steps in living cells. In this framework, fluorescence fluctuation spectroscopy is currently being explored.

Recently we investigated the interactions between the cationic polymer poly(2-dimethylamino)ethyl methacrylate (pDMAEMA) and rhodamine-labeled 25-mer phosphodiester oligonucleotides (Rh-ONs) in buffer (Van Rompaey *et al.*, 2000). The composition of the pDMAEMA/Rh-ON polyplexes was investigated as a function of the charge ratio (κ ; +/-) by increasing the pDMAEMA concentration and keeping the Rh-ON concentration constant. In dependence of the size, the zeta-potential (ζ , a measure of the potential at the hydrodynamic surface of the polyplexes) and the fluorescence of the pDMAEMA/Rh-ON polyplexes on their charge ratio, three regions could be clearly distinguished and are schematically represented in Figure 1. (i) At values of $\kappa < 1$, gel electrophoresis on pDMAEMA/Rh-ON dispersions revealed that only a part of the Rh-ON chains binds to the pDMAEMA chains. As the Rh-ONs bind to the pDMAEMA chains by only a few of their phosphate anions, the non-neutralized anions provide for the net negative

Fig. 1 Fluorescence Measured with a Conventional Fluorimeter of pDMAEMA/Rh-ON Dispersions as a Function of the Charge Ratio. The Rh-ON concentration in the dispersions was 10 $\mu\text{g/ml}$. The Rh-ON and pDMAEMA chains are represented by $\sim\bullet$ and $\sim$, respectively. The fluorescence of the Rh-ON solution in the absence of pDMAEMA (*i. e.* at $\phi = 0$) was set to 100. At values of $\phi < 1$, negatively charged particles with a diameter of around 180 nm were observed. At values of ϕ between 1 and 3, approximated zero. Dynamic light scattering revealed that in this region fully aggregated polyplexes ($> 1\mu\text{m}$) were formed. At values of $\phi > 3$, the polyplexes became positively charged having a size of around 180 nm. As the molar mass of an individual polyplex has to be in the order of some hundreds of thousands g/mol and since the hydrodynamic diameters measured in the dispersions were around 180 nm, this suggested that aggregation of individual polyplexes occurs by *e. g.* Rh-ONs that bridge individual polyplexes (as schematically represented in the Figure).

charge of the polyplexes. At $\phi < 1$, the gradual decrease of the fluorescence of the polyplex dispersions upon increasing the amount of pDMAEMA was explained by self-quenching of the fluorescence, because, upon binding the ONs significantly concentrate. (ii) At values of ϕ between 1 and 3, dynamic light scattering revealed that fully aggregated polyplexes were formed. As in this region ϕ approximated zero, the clustering of the individual polyplexes was attributed to the absence of any repulsion between the particles. (iii) At values of $\phi > 3$, the polyplexes became positively charged while electrophoresis data indicated that all the Rh-ONs were bound. As in this region the fluorescence of the dispersions again increased it was suggested that, when a higher number of pDMAEMA chains are present, the average number of Rh-ONs bound to one chain begins to decrease which results in less quenching of the fluorescence and even enhancement of the intrinsic fluorescence.

This report aims at evaluating whether the complexation between Rh-ONs and pDMAEMA could be observed by fluorescence fluctuation spectroscopy. Especially, we are interested in assessing which method of fluorescence fluctuation analysis allows us to extract information on

the pDMAEMA/Rh-ON polyplexes. Besides correlation analysis of the fluorescence fluctuations, which distinguishes between different species based on their temporal behavior, we evaluated to what extent the fluorescence fluctuation profiles could be analyzed by methods that distinguish between different species based on their difference in fluorescence.

FCS was applied for the study of the free Rh-ONs. For the analysis of the polyplexes, we first applied photon counting histogram analysis (PCH) as recently described by Chen *et al.* (1999). Briefly, PCH analysis is based upon the fact that by diffusion of fluorescent molecules through the excitation volume, the histogram of the measured photon counts exhibits a super-Poissonian behavior, fully characterized by the fluorescence signal per molecule and the quantity of all species in the sample. Crucial for extracting the molecular brightness (Φ) is the broadening of the histogram compared to a Poisson distribution.

Second, we applied the method as described by Van Craenenbroeck *et al.* (1999) in which a fluorescence threshold is defined; the fluorescence intensities larger than this threshold are considered to be signals from bright species

Results and Discussion

Figure 2 compares the fluorescence fluctuations of a 3 nM Rh-ON solution before (A) and after complexation with pDMAEMA (B, C) as measured with Confocor I. The diffusion coefficient of free Rh-ON, as calculated from the autocorrelation curve obtained from the fluorescence fluctuation profile in (A), equaled $1.05 \pm 0.02 \times 10^{-6} \text{ cm}^2/\text{s}$ (data not shown). The association of the Rh-ONs with pDMAEMA clearly influences the fluorescence fluctuation profile. The fluorescence intensity significantly decreases which agrees with the fluorimetric observations shown in Figure 1. Additionally, highly intense fluorescence peaks become visible upon complexation of Rh-ON to pDMAEMA. As multimolecular pDMAEMA polyplexes (*i. e.* several Rh-ONs per pDMAEMA chain) are suggested from Figure 1, it is very likely that the highly intense fluorescence peaks originate from the passage of pDMAEMA chains that bear several Rh-ONs. The heterogeneous distribution of the height of the highly intense fluorescence peaks probably indicates that polyplexes with a polydisperse composition, with regard to the number of Rh-ONs per polyplex, are formed. However, one cannot state that an increase in the height of the highly intense fluorescence peaks by *e. g.* a factor of two is attributed to a doubling of the number of Rh-ON per complex, because the fluorescence of pDMAEMA/Rh-ON complexes may be influenced by several factors. Also, considering the inhomogeneous excitation profile in the confocal volume, the fluorescence of a polyplex as detected by the photon detector depends on the way it moves through the confocal volume. An event in which a relatively bright complex enters the periphery of the laser beam may not be distinguished from an event in which a less fluorescent complex passes through the focus (Kask *et al.*, 1999).

Although the highly intense fluorescence peaks indicate the association of Rh-ONs with pDMAEMA chains, they greatly disturb the determination of the autocorrela-

Fig. 2 Fluorescence Fluctuation Profile (Measured by Confocor I) for a Rh-ON Solution (A) and pDMAEMA/Rh-ON Dispersions (B: $n = 8$ and C: $n = 17$). The Rh-ON concentration was $0.025 \mu\text{g}/\text{ml}$ (3 nM).

Fig. 3 Fluorescence Fluctuation Profile as Measured by PCH Setup.

8 nM Rh-ON solution (A) and the corresponding photon counting histogram (B). (C) shows the residuals between the data and the fit of the PCH (displayed in units of standard deviations).

tion function $G(\tau)$ and, consequently, no quantitative information on the Rh-ONs/pDMAEMA interactions could be obtained by correlation analysis of the fluorescence fluctuation profiles. For this reason we were forced to analyze the fluorescence fluctuation profiles in an alternative way.

Analysis of the photon counting histogram offers a way to distinguish between different species based on the difference in molecular brightness of the species and not on the temporal behavior of the fluctuations as in autocorrelation analysis (Chen *et al.*, 1999b; Müller *et al.*, 2000). The PCH is experimentally determined from the fluorescence fluctuation profile and shows the probability to detect k photons per sampling time. For a first PCH analysis we measured the fluorescence fluctuation profiles of the polyplexes on the PCH-instrument using a sampling time of $50 \mu\text{s}$. Compared with the Confocor I measurements, which always resulted in fluorescence fluctuation profiles of 509 data points (consequently, the integration time in Figure 2 is around 100 milliseconds for 50 s measuring time), the data acquisition card in the two-photon instrument allows access to the complete time resolved sequence of the photon events (Eid *et al.*, 1999). Figure 3A shows a time-averaged trace of the photon counts with an integration time of 1.6 s of an 8 nM Rh-ON solution (in the absence of pDMAEMA) as measured by the two-pho-

ton excitation setup. The symbols connected by the solid line in the semilogarithmic plot of Figure 3B show the corresponding photon count distribution for a sampling time of 50 μ s. Based on the computer algorithm developed by Chen *et al.* (1999a), which fits the experimental PCH, we were able to extract the average number of Rh-ONs in the observation volume (N) and the molecular brightness of the Rh-ONs from the fitting. The fit determined N to be 0.66, while γ equaled 5200 cpsm (counts per second per molecule). As Figure 3C shows, the residuals between the data and the fit of the PCH (displayed in units of standard deviations) vary randomly and yield a reduced X^2 close to 1, indicating a good description of the data by a single-species model.

Figure 4A shows an integrated time trace of the photon counts upon complexation of Rh-ONs to pDMAEMA ($\gamma = 1$) measured by the PCH setup. As observed in the sin-

gle-photon measurements on Confocor I (Figure 2), the fluorescence intensity decreases while highly intense fluorescence peaks arise. The highly intense fluorescence peaks affect the PCH at higher counts (Figure 4B). By fitting these data to a two-species model, we resolved 2 species with totally different molecular brightness values: a rather dim species with $\gamma_1 = 5200$ cpsm ($N_1 = 0.69$), which corresponds to the free oligonucleotide, and a much brighter species with $\gamma_2 = 70\,000$ cpsm ($N_2 = 0.001$), the multimolecular complex. However, a strictly quantitative interpretation of the PCH parameters for the very bright species remains problematic. The difficulty lies in the 'rarity' of the events associated with the bright species and the heterogeneity of the formed pDMAEMA/Rh-ON complexes. To illustrate this point, consider the fluctuation trace in Figure 4A. If we split the data around 400 seconds into two sets (set 1 and set 2) and

Fig. 4 Fluorescence Fluctuation Profile of a pDMAEMA/Rh-ON Dispersion Measured with the PCH Setup.

The dispersion have a value of $\gamma = 1$ (A); the corresponding photon counting histogram with experimental data (■) and PCH fit (●) is shown in panel (B). The Rh-ON concentration was 8 nM. The rectangle shows the fluorescence data, between two highly intense fluorescence peaks, used for PCH analysis. (C) shows the residuals between the data and the fit of the PCH (displayed in units of standard deviations).

analyze each sequence individually, we arrive at the same result for the dim species. However, for the bright species the number of molecules and the brightness differ in both cases (data not shown). This fact is readily explained by visual inspection of Figure 4A: no strong intensity fluctuations are observed for the first 400 seconds, while a few events are visible in the later part of the fluctuation trace. The molecular brightness obtained by PCH analysis of the fluorescence fluctuations between highly intense fluorescence peaks (the fluctuations indicated by the rectangle in Figure 4) allowed us to conclude that these fluctuations originated from the diffusion of the free Rh-ONs as (5000 cpsm) agreed quite well with of free Rh-ONs as obtained from Figure 3. Figure 5A presents an integrated time trace of photon counts of a pDMAEMA/Rh-ON dispersion with $\alpha = 4$. The fluorescence intensity further decreases while the amount of highly fluorescence

peaks significantly increases which might indicate that the number of polyplexes increases. Despite the larger number of highly intense fluorescence peaks, the interpretation of the PCH analysis remains difficult. Probably the sample is very heterogeneous and even if many events are observed, the number of events per particular polyplex is still rare. The insert in Figure 5 shows the complete time resolved sequence of the photon events underlying one fluorescence peak in the integrated time trace of photon counts. The peak in the integrated time trace seems to originate from the passage of one bright pDMAEMA/Rh-ON complex. Remarkable is the long residence time of the complex in the confocal volume, almost 500 ms. Also interesting is the fine structure in the pattern; upon passing through the confocal volume, at a certain moment the fluorescence significantly drops. Three hypotheses may explain this pattern: (i) although

Fig. 5 Fluorescence Fluctuation Profile of a pDMAEMA/Rh-ON dispersion Measured with the PCH Setup.

The dispersion had a value of $\alpha = 4$ (A) and the corresponding photon counting histogram with experimental data (■) and PCH fit (●) (B). The Rh-ON concentration equaled 8 nM. This insert shows the complete time resolved sequence of the photon events of the fluorescence peak that appeared at 183 s in the integrated fluctuation profile. (C) shows the residuals between the data and the fit of the PCH (displayed in units of standard deviations).

Fig. 6 Fluorescence Fluctuation Profile Measured by PCH Set-up of the pDMAEMA/Rh-ONs Dispersion with a Value for $\tau = 1000$.

The Rh-ON concentration was 8 nM.

highly unlikely, immediately after the passage of a polyplex through the confocal volume a second polyplex may come in; (ii) considering the dynamic features of the polyplexes, quenching and dequenching phenomena in the polyplex may cause blinking of the complex while it stays in the confocal volume; (iii) the polyplex may pass through the focus, subsequently it may move toward the periphery of the confocal volume element and may move back into the focus.

To rule out optical laser trapping as a source of the long residence time of particles in the observation volume, we repeated the same experiments at lower laser powers. We observed qualitatively the same features and residence times as at the higher laser power; a strong argument against a mechanism based on laser trapping.

As explained by Figure 1, at a high excess of pDMAEMA (*i. e.* at extremely high values of τ) one expects the presence of monomolecular (one Rh-ON per pDMAEMA chain) instead of multimolecular complexes. This was indeed observed in the fluorescence fluctuation profile of pDMAEMA/Rh-ON dispersions with $\tau = 1000$ (Figure 6) which did not show highly intense fluorescence peaks. This allowed PCH analysis with a single species model, revealing values for N and σ of respectively 0.52 and 8700 cpsm. Remarkably, the value for σ of such monomolecular pDMAEMA/Rh-ON complexes was higher compared with σ of the free Rh-ON (5200 cpsm). The increase in σ agrees with the increase in the fluorescence intensity of the fluctuation profiles (from 3500 Hz in Figure 3 to 4600 Hz in Figure 6) and was also reflected in the fluorescence of the polyplexes as measured using a conventional fluorimeter (Figure 1); the fluorescence of the pDMAEMA/Rh-ON complexes at $\tau = 20$ (Figure 1) was higher than the fluorescence of the free Rh-ON solution. Although the reason is unclear, the presence of the pDMAEMA chains results in a changed environment of the Rh-ONs, which may alter their fluorescence properties. Several groups reported that the fluorescence of dyes such as 5-carboxytetramethylrhodamine could be partially quenched

upon binding to an oligonucleotide, the degree of quenching being dependent on the fluorophore's proximity to purines and its position in the oligonucleotide (Hawkins *et al.*, 1995; Vamosi *et al.*, 1996). It is hypothesized that the pDMAEMA chains might lead to a 'de-quenching' of the rhodamine molecules bound to the ONs (the increased intensity corresponds to an increased average fluorescence lifetime; (results not shown)). As it remained difficult to extract information on the complexation between Rh-ONs and pDMAEMA from PDH analysis, we looked further for alternative ways to analyze the fluorescence fluctuation profiles. Recently, Van Craenenbroeck *et al.* (1999) proposed a new method to analyze fluorescence fluctuation profiles of a mixture of different types of fluorescent molecules where one type of the molecules shows much brighter fluorescence compared to the other. This method is applicable when integration is done over long time periods, such that a single species gives rise to a Gaussian distribution of the intensity fluctuations (Van Craenenbroeck *et al.*, 1999). Based on this observation, an heuristic method was developed that allows to discriminate between the fluorescence intensities belonging to the Gauss distribution of the unbound species and the bright spikes belonging to the multiplexes. The Kolmogorov-Smirnov goodness of fit test is used to determine the threshold level necessary for this discrimination. The nonlinear nature of this analysis can give an enhanced sensitivity by effectively separating the signal contribution of the bright complexes from the dim fluorophores. The signal statistics of photon counts is a complex issue and incorporating the effects of the nonlinear operation is not a trivial matter. We applied this type of analysis to the fluorescence fluctuation profile of the pDMAEMA/Rh-ON dispersions measured by the Confocor I setup. When only free Rh-ONs were present, the fluorescence intensities in the fluorescence histogram showed a Gaussian distribution, typical for data recorded in longer time bins (data not shown). However, the intensity of the highly intense fluorescence peaks (regarded as originating from pDMAEMA/Rh-ON complexes) as ob-

Fig. 7 Number of Highly Intense Fluorescence Peaks Observed in the Fluorescence Fluctuation Profile of the pDMAEMA/Rh-ON Dispersions (Measured with Confocor I) as a Function of the Charge Ratio.

served in the fluorescence fluctuation profile of the pDMAEMA/Rh-ON dispersions whose intensity no longer showed a Gaussian distribution (data not shown). Figure 7 shows the number of the highly intense fluorescence peaks in the fluorescence fluctuation profile of the pDMAEMA/Rh-ON dispersions. For pDMAEMA/Rh-ON complexes having α -values lower than 15, increasing resulted in an increase in the number of highly intense fluorescence peaks. As the Rh-ON concentration was the same in all the pDMAEMA/Rh-ON dispersions, a higher number of pDMAEMA chains will decrease the average number of Rh-ON bound per pDMAEMA chain as discussed above. This will increase the number of polyplexes, which explains the increase in the number of highly intense fluorescence peaks. However, upon further increasing the number of pDMAEMA chains one could expect that, from a certain value of α , polyplexes would arise that consist of only one or a few Rh-ONs bound per pDMAEMA chain and whose fluorescence intensity again belong to the Gaussian distribution. Such polyplexes would not result in highly intense fluorescence peaks in the fluorescence fluctuation profile and, as observed, it is expected that the number of highly intense fluorescence peaks will decrease.

In conclusion, complexation between Rh-ONs and pDMAEMA can be observed by fluorescence fluctuation spectroscopy although it is hard to obtain quantitative information. When intensity bursts appear in the fluctuation profile, due to the presence of multimolecular complexes, the interpretation of the PCH analysis remains difficult. However, the method of Van Craenenbroeck *et al.* (1999) allows us to determine the number of fluorescence bursts that can be considered as a relative amount of the number of multimolecular complexes. When highly intense fluorescence peaks do not appear in the fluorescence fluctuations, indicating the existence of monomolecular complexes, the method of Van Craenenbroeck *et al.* is not applicable, whereas PCH analysis can be applied to determine the molecular brightness and the number of these polyplexes in the confocal volume.

Materials and Methods

Oligonucleotides

The 25-mer rhodamine labeled phosphodiester ONs (5'-TCT-GGG-TCA-TCT-TTT-CAC-GGT-TGG-C-3') (Rh-ONs) were purchased from Eurogentec (Seraing, Belgium). The fluorescent labeling occurred at the 5' end of the ONs with 5-(and-6)-carboxytetramethylrhodamine (succinimidyl ester); each Rh-ON molecule contained one Rh label. The molecular weight of the Rh-ONs was 8253 g/mol.

Cationic Polymers

pDMAEMA was synthesized and characterized as described elsewhere (van de Wetering *et al.*, 1997). The molecular mass of the DMAEMA monomer was 157 g/mol. The average molecular mass (M_n) of pDMAEMA was estimated to be around 60 000 g/mol.

Preparation of Polyplexes

For fluorescence fluctuation spectroscopy, pDMAEMA/Rh-ON complexes differing in α , but having the same Rh-ON concentration (8 nM) were prepared. α , being the charge ratio (+/-) of the positive charge equivalents on pDMAEMA to the negative charge equivalents on Rh-ON, was calculated assuming that 1 μ g of 25 mer Rh-ON contains 2.91 nmol negative charges and 1 μ g of pDMAEMA contains 3.54 nmol positive charges as calculated from the molecular weight of the DMAEMA monomer and the pKa of the pDMAEMA.

Particle Size, Zeta Potential and Fluorescence Measurements on the Polyplexes

Dynamic light scattering experiments on the polyplexes to determine their particle size were carried out on a Malvern 4700 instrument (Malvern, Worcestershire, UK) at 25 °C and at an angle of 90 °. The incident beam originated from a HeNe laser (633 nm).

Zeta potential measurements on the polyplexes were performed at 25 °C on a Malvern Zetasizer 2000 (Malvern). This instrument measures the velocity of a particle in an electrical field using a light scattering technique. Due to the Doppler effect the frequency of the scattered laser light is different from the frequency of the original laser beam. The frequency shift is related to the electrophoretic mobility u and, consequently, to the charge properties of the complexes. The zeta potential was calculated by the Smoluchowski equation:

$$\zeta = 4 (\eta u) / \epsilon_s$$

where η is the viscosity of the solution and ϵ_s is the dielectric constant of the solvent.

The fluorescence of pDMAEMA/Rh-ON complexes was measured 30 min after their preparation on an SLM-Aminco bowman fluorimeter (SLM-Aminco Bowman, Rochester, NY, USA; λ_{ex} = 525 nm, λ_{em} = 584 nm). Fluorescence of each dispersion was measured three times.

Fluorescence Fluctuations Measurements

For fluorescence fluctuation experiments single-photon and two-photon excitation set-ups were used in this study. As the single-photon excitation system Confocor I (Zeiss-Evotec, Jena/Hamburg, Germany) was used. Briefly, the light of a HeNe laser operating at 543 nm was projected into the microscope water immersion objective (C-apochromat 40x magnification) via a dichroic mirror. The laser beam was focused at about 180 μ m above the bottom of the cuvettes (Nalge Nunc International, Naperville, IL, USA). The emitted light was collected by the same objective, passed through the dichroic mirror and the 45 μ m pinhole to finally reach the avalanche photodiode. Measurements on the Rh-ON solution and polyplex dispersions were performed at room temperature on samples of 200 μ l. Each sample (differing in α) was prepared 3 times ($n = 3$) and was measured 60 times during 50 s. The integration time in the fluorescence fluctuation profiles was about 100 ms.

For the two-photon excitation measurements, a mode-locked Ti:Sapphire laser (Mira 900; Coherent Inc., Santa Clara, CA, USA) pumped by a diode-pumped intracavity doubled Nd:YVO4 Vantate laser (Verdi, Coherent Inc.) was used as the two-photon excitation source. For all measurements, an excitation wavelength of 780 nm was used. Photon counts were detected with an APD (SPCM-AQ-161; EG&G, Canada). The output of the APD unit, which produces TTL pulses, was directly connected to the data acquisition card. The recorded and stored photon counts

were analyzed with PV-WAVE version 6.10 (Visual Numerics). A Zeiss Axiovert 135 TV microscope (Thornwood, NY, USA) with a 63x Plan Apochromat oil immersion objective (NA = 1.4) was used. Measurements on the Rh-ON solution and polyplex dispersions were performed at room temperature on samples of 1 ml. Each sample (differing in λ) was prepared 3 times ($n = 3$) and the fluorescence fluctuations were recorded during 500 or 800 s for the Rh-ON solutions and polyplex dispersions, respectively. The measurements were performed in the time-mode, the photon counts were sampled at 20 kHz resulting in a sampling time of 50 μ s. The PCH analysis of the fluorescence fluctuations was performed as described in detail by Chen *et al.* (1999a).

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