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The Effect of Spermine on Plasmid Condensation and Dye Release Observed by Fluorescence Correlation Spectroscopy

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We demonstrate that fluorescence correlation spectroscopy (FCS) can be employed to follow the conformational changes of DNA molecules induced by the addition of a cationic condensing compound (spermine). In our experiments the plasmid pHβAPr-1-neo (10 kbp; contour length 3.4 μm) was labeled with propidium iodide (PrIo) and then titrated with spermine to induce its condensation. When spermine was applied at concentrations above 5 μM (spermine/DNA$_{phosphate}$ =0.375), the diffusion time of the labeled plasmid dropped from 15 ms down to 3 ms (its diffusion coefficient, D, increased from 1.0×10^{-12} m^2/s to 6.0×10^{-12} m^2/s). The application of spermine was also accompanied by decreasing count rate and particle number, reflecting the dye's dissociation. The data presented show that FCS may become a valuable tool in studying supramolecular aggregate formation, especially when association is followed by a change in the hydrodynamic size of the resulting complex.

Key words: Cationic compounds / Confocal microscopy / DNA condensation / DNA conformation / FCS / Propidium iodide.

Introduction

Clinical application of gene therapy requires the development of efficient and well-characterized supramolecular carriers. Problems associated with constructing safe and immuno-neutral viral formulations have triggered the intensive research of artificial structures (Huang *et al.,* 1997; Fattal *et al.,* 1999; Langner 2000). Various aggregates based on polymers, peptides and lipids have been developed (Garnett, 1999; Kabanov, 1999; Pedroso-de-Lima *et al.,*1999). However, mainly due to the lack of understanding DNA-carrier aggregate formation mechanisms, these carriers had unpredictable and inhomogeneous final structures, thus resulting in low transfection efficiency (Langner, 2000).

Various techniques have been used to determine the properties of such aggregates and the processes leading to their formation. Most of them rely on the averaged parameters measured throughout an entire sample (Eastman *et al.,* 1997; Radler *et al.,* 1997; Anchordoquy *et al.,* 2000; Lobo *et al.,* 2001; Matsui *et al.,* 2001). Such an approach does not allow, for example, the discrimination of homo- and heterogeneous populations. On the other hand, single molecule approaches (including microscopy) require time-consuming protocols in order to obtain representative population distributions (Mel'nikov *et al.,* 1995a, b, 1997; Mel'nikova *et al.*, 1999). For these reasons, a technique able to overcome those obstacles is needed. Here we show that FCS may enable fast acquisition of data concerning aggregate formation on the single molecule level.

The wide use of fluorescence detection and DNA imaging stimulated the development of a variety of fluorescence probes (Haugland, 1996). Two of them, ethidium bromide and propidium iodide, are particularly popular. These dyes increase their fluorescence upon association with DNA. However, this is an increase of only an order of magnitude, causing background level fluorescence to be high and potentially bias measured values. Furthermore, the probe's association efficiency is likely to depend on DNA conformation and the presence of other molecules able to interact with DNA. Such interference can be safely neglected for imaging purposes, but not when single molecule detection is needed or when processes associated with the condensation of genetic material are studied. We demonstrate that FCS can be successfully employed to measure the condensation of propidium iodide-labeled DNA induced by spermine (Bloomfield, 1991, 1998).

Results and Discussion

In order to study the DNA condensation process, the 10 kpb plasmid pHβAPr-1-neo was used. The plasmid was pre-labeled with a constant molar fraction of propidium

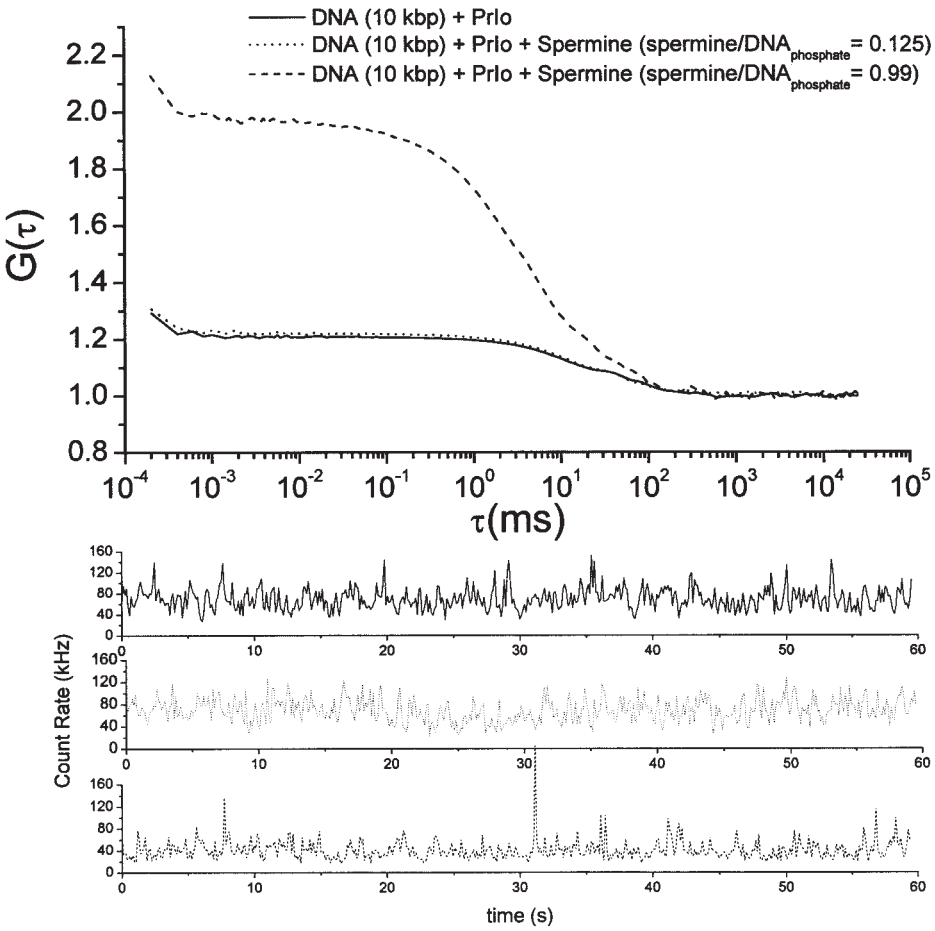

Fig. 1 Normalized Fluorescence Autocorrelation Functions.
Data were obtained for plasmid pHβAPr-1-neo (10 kbp; contour length 3.4 μm) labeled with propidium iodide measured alone (continuous line) and at two spermine/$DNA_{phosphate}$ ratios, 0.125 (dashed line) and 0.99 (dotted line). The $C_{DYE}/C_{phosphate}$ ratio was equal to 0.04. Corresponding fluorescence count rates are shown in the lower panels.

iodide (PrIo) ($C_{DYE}/C_{phosphate}$=0.04) and titrated with spermine. Following each addition of the condensing agent, fluorescence intensity and count rate were measured. After deriving the autocorrelation function, the diffusion time, count rate (CR) and particle number (PN) were calculated. Typical count rates and corresponding normalized autocorrelation functions are presented in Figure 1. These three recordings reveal the extent of change that occurs upon adding two spermine concentrations: one that does not induce DNA condensation (spermine/$DNA_{phosphate}$ ratio=0.125) and one that does (spermine/$DNA_{phosphate}$ ratio=0.99). When the diffusion times of the plasmid alone (15 ms) and of plasmid with a condensing spermine concentration (3 ms) are compared, a drastic difference of 12 ms is evident. The dependence of the diffusion constant (easily calculated from diffusion time) on spermine concentration is shown in Figure 2A, from which the concentration of spermine required to condense the plasmid can be estimated. With our experimental setup, it turned out to be approximately 12 μM. The corresponding spermine/$DNA_{phosphate}$ ratio (0.45) complies with literature data, validating the measurements of such macromolecular systems with the FCS technique (Delcros *et al.,* 1993).

When the spermine concentration remains below 6 μM (spermine/$DNA_{phosphate}$<0.5), DNA molecules remain in the uncondensed state; this is reflected by constant diffusion time, count rate and particle number. In such a concentration range, correlation curves are well fitted by equation 1 with the assumption that a single diffusing species is present in the sample. In the transition region (0.5<spermine/$DNA_{phosphate}$<0.8) all three parameters change drastically, indicating that condensation occurs. The use of a model assuming two discrete particle classes, however, did not significantly improve the fit to the data when compared with the one component model (eq. 1), possibly indicating a rather broad size distribution. At spermine/$DNA_{phosphate}$ ratios larger than 0.8, diffusion time, count rate and particle number remain again constant. At this concentration the detectable DNA population appears to be rather uniform regarding its hydrodynamic properties. Diffusion time of the condensed particles was reproducible in a series of independent experiments, with a standard deviation of 20%. Such a standard deviation agrees with results obtained for other supramolecular assemblies with similar diffusion constants (Beneš *et al.*, 2002).

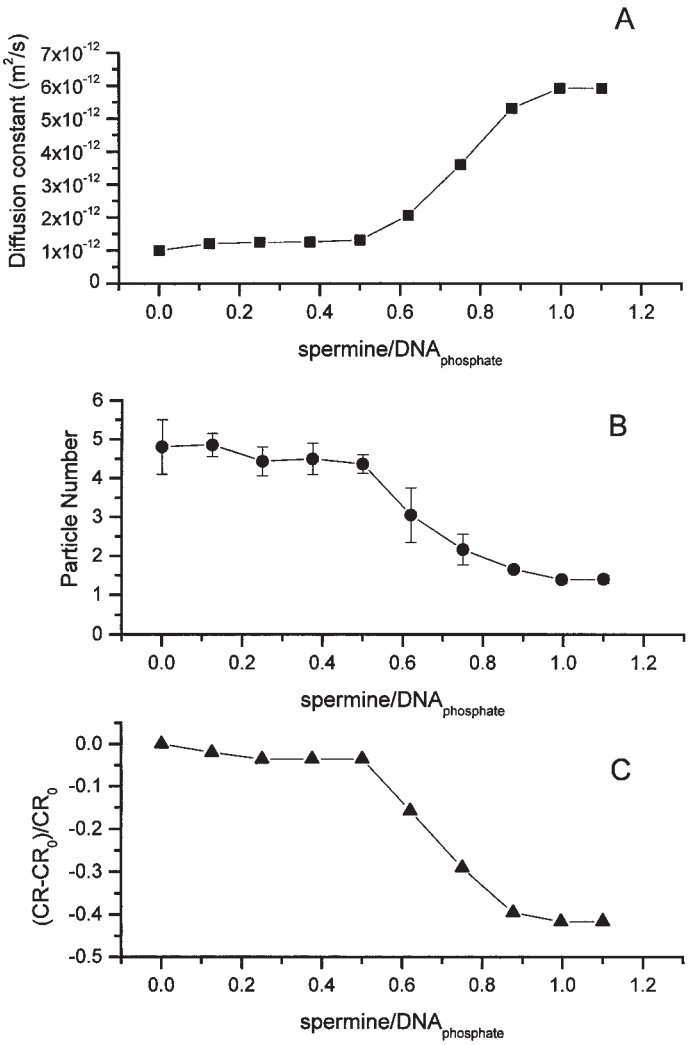

Fig. 2 The Dependence of the Diffusion Constant (D_t; Panel A), Particle Number (Panel B) and Relative Count Rate [$(CR–CR_0)/CR_0$; Panel C] of Plasmid pHβAPr-1-neo on the Spermine/$DNA_{phosphate}$ Ratio.
The $C_{DYE}/C_{phosphate}$ ratio was 0.04.

As shown in Figure 2, panels B and C, increase of the diffusion constant is paralleled by a decrease of count rate and particle number. The midpoints of both titration curves appear to be at similar spermine concentrations, as in the case of the diffusion coefficient dependence. The drop of relative count rate indicates that fluorescent molecules dissociate from their DNA binding sites upon spermine addition. The drop of particle number means that the majority of DNA molecules are not sufficiently labeled, making them undetectable in the FCS experiment. As a result, the amount of unbound propidium iodide increases dramatically when condensing concentrations of spermine are employed. The presence of unbound fluorescent dye at μM concentrations of leads to significant fluorescence background. Free floating dye molecules then exceed the number of DNA aggregates by of few orders of magnitude, making them undetectable as separate events. Hence they do not contribute to the measured particle number, but are still fluorescent. This situation causes the apparent particle brightness to increase when the number of visible DNA molecules decreases.

A comparison of the FCS data presented here and available fluorescence microscopic measurements shows that the two techniques lead to qualitatively similar conclusions regarding the condensation process. Results presented by Mel'nikov *et al.* (1995a) reveal that population-wise, the condensation process progresses gradually, with the coexistence of both the globular and coil DNA forms in a well-defined condensing agent concentration range. Below and above this range only uniform particles are observed. Similar conclusions can be drawn from data presented in Figure 2.

A decrease of count rate may result from the quenching of propidium iodide fluorescence by rising spermine concentration. In order to exclude this possibility, separate steady-state fluorescence measurements have been performed. Complexes of propidium iodide and nucleic acid of low molecular weight (twenty bases) were titrated with an appropriate amount of spermine. Short chain

oligonucleotides (bellow 400 bases) do not change their conformation upon interaction with counterions (Bloomfield, 1998), therefore any changes in fluorescence intensity indicate dye-titrant interactions. An increase in propidium iodide fluorescence intensity upon the addition of oligonucleotide proved that the dye and nucleic acid associate. Titration with spermine, however, did not cause any change in dye fluorescence intensity, showing that the quenching effect is not present (data not shown). Based on this experiment, we concluded that the observed decrease of count number results from dye desorption from the DNA molecule rather than from other factors (Figure 2C).

The data presented here show that the FCS technique is a valuable tool in studying aggregation processes, especially when the sample tends to have an inhomogeneous particle distribution. The DNA condensation process is a complex event that depends on a variety of factors, including DNA molecule state, condensing agent chemical structure, aqueous phase properties and sample history. It has been shown that spermine, which is known to interact strongly with nucleic acid, efficiently induces condensation that results in a tightly packed uniform population of aggregates. FCS experiments provide results that are in good agreement with available literature data, including the spermine concentration required to collapse plasmid. It needs to be emphasized that FCS provides information at the single molecule level. Combined with the analysis of a sufficiently large particle population it enables the simultaneous investigation of sample homogeneity and single particle behavior. Furthermore, the method's sensitivity allows for lower DNA and fluorescent label concentrations, which reduces the danger of concentration-dependent particle aggregation and fluorescent probe interference in the followed processes. This is especially important if the fluorescent label binds strongly to the DNA molecule, such as YOYO, and events involving nucleic acids need to be quantitatively evaluated. This aspect is of high relevance when DNA stains are employed to monitor exogenous genetic material fate within the cell*, i. e.* fluorescence microscopy or FACS.

In summary, despite a number of yet unresolved technical difficulties, the FCS technique proves to be a valuable tool for studying supramolecular ensembles, with the unique capability of combining both single molecule detection and particle population analysis.

Materials and Methods

Spermine was purchased from Sigma (Atlanta, GA, USA) and propidium iodide (PrIo) from Molecular Probes (Eugene, OR, USA). pHβAPr-1-neo plasmid (10 kbp and contour length 3.4 μm) was a generous gift from laboratory of Dr. Maciej Ugorski Laboratory (Department of Immunochemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland). The plasmid was prepared according to methods described elsewhere (Sambrook *et al.,* 1989), with slight modifications during the final purification stage. Namely, DNA was precipitated with isopropanol, the sample centrifuged at 12 000 *g* for 5 min at room temperature. The pellet was dissolved in a solution of RNase A (10 μg/ml) and incubated for 15 min. Plasmids were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and twice with chloroform:isoamyl alcohol (24:1, v/v). The combined upper phases were precipitated with 5 M of potassium acetate:isopropanol:water (1:22:2, v/v/v). After centrifugation, the DNA pellet was washed with 70% ethanol and re-dissolved in water.

Fluorescence measurements were performed on a ConfoCor® 1 (Carl Zeiss Jena, Germany). ConfoCor 1 is a PC-controlled fluorescence correlation-adapted AXIOVERT 135 TV microscope, equipped with an x-y-z adjustable pinhole, avalanche Photodiode SPCM-200-PQ, ALV-hardware correlator, and CCD camera. The Ar^+-laser beam (excitation wavelength: 514 nm; excitation intensity: 1 mW) was focused by a water-immersion microscope objective (NA=1.2) at an open focal light cell. This objective, a dichroic mirror, proper bandpass filters and a pinhole in the image space block collected fluorescence light. The size of the confocal volume element, calibrated with rhodamine-6G, was determined to be about 1 fl.

Fluorescence intensity I(t) fluctuations around a temporal average, $I(t)=\langle I(t)\rangle+\delta I(t)$, were processed using a digital hardware correlation interface, which yielded the normalized autocorrelation function G(t) to be $G(t)=1+\langle \delta I(t)\delta I(t+\tau)\rangle/\langle I(t)\rangle^2$. The general solution of the three-dimensional autocorrelation function G(t) for translational diffusion in an ellipsoid confocal volume is:

$$G(t) = 1 + \frac{1}{N} \cdot \frac{1}{1+(t/\tau_D)} \left\{ \frac{1}{1+(t/\tau_D)\,(\omega^2_1/\omega^2_2)} \right\}^{1/2} \qquad (1)$$

where ω_1 is the volume element radius in the xy plane, ω_2 its half-length in the z direction and N particle number. When ω_1 and τ_D are known, the diffusion coefficient (D) can be determined as $\tau_D=\omega_1^2/4D$. All calculations were performed with the assumption that fluorescence decay (τ_f) and translational diffusion τ_d were well separated in time ($\tau_f<<\tau_d$).

The theoretical fitting function (eq. 1) needs to be corrected for an average fraction of dye molecules in the triplet state and for the intersystem crossing relaxation time effect. Molecules in the triplet state are considered to be non-fluorescent. When the theoretical expression does not fit satisfactorily to experimental data, a term for a second diffusing species labeled with the same fluorophores was added. A detailed description of this theoretical analysis is given in several review articles (Thompson, 1991; Schwille *et al.,* 1997) and in our recent publication (Beneš *et al.,* 2001).

Samples for FCS measurement were prepared as follows: an appropriate amount of aqueous DNA solution (ranging from 1 nM to 2 nM) was placed into the chamber with the glass bottom facing the ConfoCor water-immersion microscope objective. The fluorescent label was then added from a water stock solution (10 μM), and this was titrated with the condensing agent. Fluorescent probe concentration was predetermined by spectroscopic measurements and adjusted to obtain the final $C_{Dye}/C_{phosphate}$ ratio of 0.04. All experiments were carried out at room temperature in eight-chamber cover glasses (NUNC®). Experimental data were fit by FCS ACCESS evaluation software (1-component fitting, with triplet state consideration). All samples were measured in distilled and deionized water (pH 6.85).

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