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FLUORESCENCE CORRELATION SPECTROSCOPY (FCS) AS A TOOL TO STUDY DNA CONDENSATION WITH HEXADECYLTRIMETHYLAMMONIUM BROMIDE (HTAB)

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Abstract: The presented data show that the FCS technique can be used to detect the DNA condensation process induced with the cationic compound hexadecyltrimethylammonium bromide (HTAB). We have shown that HTAB induces plasmid condensation upon interaction with it. Condensation can be considered to be complete when the diffusion constant reaches its maximum. The HTAB induced increase in diffusion time does not correlate well with the changes observed when count rate and particle number are considered. This observation contradicts data published for another cationic agent, spermine. This apparent discrepancy proves that the mechanisms of interaction between these compounds and DNA are different. Consequently, the different characters of the plots of count rate, diffusion time, and particle number versus condensing agent concentration can be a source of additional information about the nature of cationic compound-DNA interaction.

Key Words: Condensation, DNA, Cationic Compound, FCS

INTRODUCTION

DNA conformation has been subject to intensive research throughout recent decades. A variety of experimental techniques and theoretical studies have been employed to describe the interaction of DNA molecules with many compounds,

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ranging from simple ions to proteins [1-4]. Despite enormous effort, the accuracy of predicting DNA-amphiphilic compound aggregate conformation is still very low. The prediction of aggregate topology is a key issue in designing an artificial carrier for genetic material [5-7]. Such a carrier is a prerequisite for the development and subsequent successful application of therapies that are based on genetic information. The formation of supramolecular aggregates that contain amphiphilic components depends on a variety of factors, including the intrinsic properties of amphiphilic molecules, environmental conditions and aggregate history. Aggregate formation can be considered to consist of two stages: the neutralization of DNA phosphate group negative charges with positively charged compounds, causing DNA condensation, and subsequent aggregate rearrangement determining the/an overall complex topology [8]. DNA condensation can be induced by a wide range of compounds – ions, proteins, cationic lipids and polymers. The resulting aggregate structure and stability critically depends on the amphiphilicity of the condensing agent and on DNA molecule size [9]. Since the processes that lead to DNA condensation are poorly known, they are difficult to control, and the resulting aggregates are frequently heterogeneous and unstable.

Studying such complex, multi-component systems, two general approaches are employed: whole sample analysis and single molecule studies. The first approach averages the entire particle population in a given sample, thus giving a result which is difficult to analyze due to the coexistence of a number of different aggregate populations. The second approach provides information that pertains to single molecule behavior and properties, but it requires a statistically significant number of measurements in order to obtain reliable data [10]. In studying DNA-amphiphile complexes, optimal results are obtained when both approaches are employed. The FCS technique provides the unique possibility of measuring a statistical distribution of particles that differ in their hydrodynamic size, by accumulating a large number of single molecule measurements. FCS is based on measurements of the residence time of labeled DNA molecules in a small volume, where excitation light is focused (confocal). FCS has been used to measure intermolecular interactions such as ligand-receptor binding, protein and polymer aggregation, and the diffusion of labeled molecules on the lipid bilayer plane or within the cell. The hydrodynamic sizes of relaxed and condensed DNA differ by orders of magnitude, hence observing and measuring the condensation process with the FCS technique seems feasible. In this paper, we show that it is possible to measure DNA condensation induced by a cationic compound (HTAB).

MATERIALS AND METHODS

HTAB was purchased from Sigma (Atlanta, GA, USA), propidium iodide (PrIo) from Molecular Probes (Eugene, Oregon, USA). pHβAPr-1-neo plasmid (10 kbp and 3.4 μm contour length) was donated by Dr. Maciej Ugorski's Laboratory

(Department of Immunochemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland).

Fluorescence measurements were performed on a ConfoCor® 1 (Carl Zeiss Jena, Germany). The Ar^+-laser beam (excitation wavelength – 514 nm, excitation intensity – 1 mW) was focused using a water-immersion microscope objective (NA = 1.2) on an open focal light cell. The confocal volume element, calibrated with rhodamine-6G, was determined to be about 1fL.

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

$$G(t) = 1 + \frac{1}{N} \cdot \frac{1}{1 + (t / t_D)} \left\{ \frac{1}{1 + (t / t_D)(w_1^2 / w_2^2)} \right\}^{1/2} \quad (1)$$

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 ($\tau_f << \tau_d$).

A detailed description of this theoretical analysis is given in several review articles [11, 12] and in our recent publications [13-16].

Samples were prepared as follows: an appropriate amount of the solution (DNA in deionized water) was placed into the chamber, and titrated with the condensing agent after the addition of a fluorescent label (PrIo). Fluorescent probe concentration was predetermined by spectroscopic measurements and adjusted to a final $C_{dye}/C_{DNA\ phosphate}$ ratio of 0.04. The experimental data were fit by FCS ACCESS Evaluation software (1-component fitting with triplet state consideration).

RESULTS AND DISCUSSION

Plasmids were labeled with the fluorescent dye propidium iodide (PrIo), which associates with the double helix. The fluorescence intensity of PrIo increases upon binding to DNA, whereas its fluorescence intensity in the aqueous phase is much lower. Consequently, fluorescence originating from the sample originates predominantly from the bound dye fraction. Unbound dye contributes to the overall fluorescence (count rate), but dye molecules are too numerous to be detected as a separate event. Thus, it does not contribute to the calculated

particle number and diffusion constant. The 10 kbp fluorescently labeled plasmid in water was titrated with a cationic amphiphilic compound (HTAB), which, upon interacting electrostatically with DNA phosphate groups, should induce its collapse. The cationic compound competes with the fluorescent dye for binding seats, forcing its desorption. Consequently, the extent of dye desorption (accompanied by decreasing fluorescence intensity) can be used as a measure of cationic compound-DNA complex formation. Due to the reduced hydrodynamic size, plasmid condensation should be accompanied by a drastic reduction of the DNA molecule's diffusion constant. Both parameters can be simultaneously detected with FCS in a single experiment.

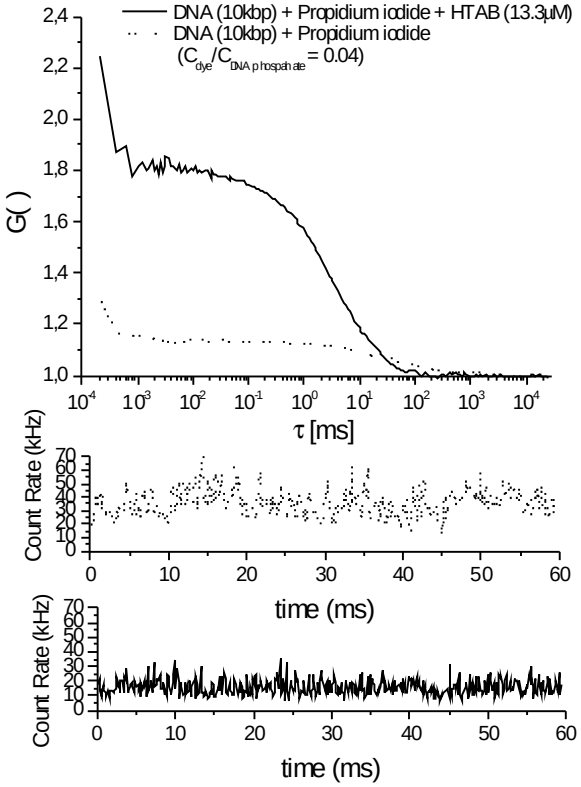

Fig. 1. The upper panel shows the autocorrelation function obtained for circular plasmids labeled with propidium iodide, with (dotted line) and without (continuous line) 13.3 μM HTAB added. Corresponding count rates are shown on the lower panels.

Figure 1 shows representative fluorescence intensity fluctuations and the corresponding autocorrelation function of plasmid with and without HTAB. Fluorescence intensity fluctuations for the plasmids alone are significantly smaller than those in the presence of the cationic compound (HTAB).

The autocorrelation function allows the calculation of three parameters: diffusion time (the diffusion constant), particle number and count rate. Particle number is a straightforward parameter that reflects the number of labeled particles. Diffusion time can be correlated with particle hydrodynamic size, and count rate is a measure of the amount of fluorescence coming from the sample (both from free and bound dye). Figure 2A shows the diffusion constant as a function of HTAB concentration. Up to 6 μM of HTAB the diffusion constant remains around 10^{-12} m^2s^{-1}. Above this concentration it begins to rise, eventually reaching a level of 4.5×10^{-12} m^2s^{-1} and reflecting the reduction of plasmid hydrodynamic size. The dependence of the diffusion constant on HTAB concentration has a sigmoidal character, indicating the high cooperativity of the condensation process.

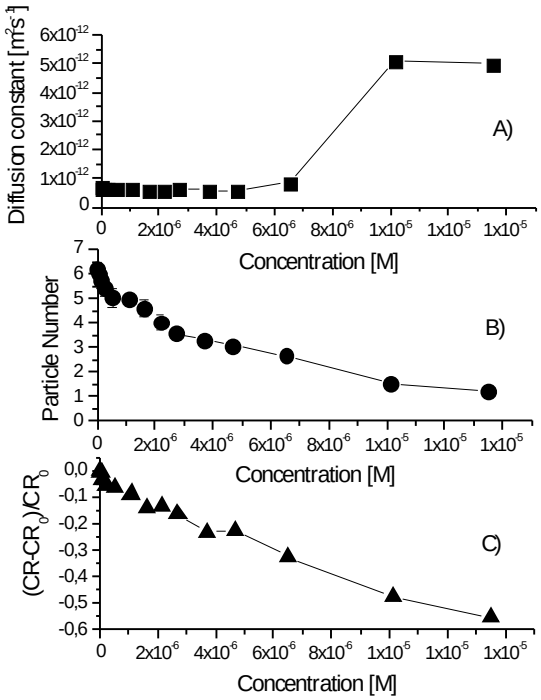

Fig. 2. The dependence of the diffusion constant (A), particle number (B) and relative count rate (C) on HTAB concentration for samples containing plasmid labeled with propidium iodide ($C_{PrIo}/C_{DNA\ phosphate}$ = 0,04).

The dependence of particle number on HTAB concentration has a different character. Particle number decreases continuously with increasing condensing agent concentration. This result shows that fluorescent dye dissociation does not correlate well with the DNA condensation indicated by the rising diffusion constant.

The number of particles possessing detectable fluorescence decreases despite the fact that the diffusion constant remains stable and follows the decreasing count rate closely.

The interpretation of such data is not straightforward. When the same plasmids were condensed with spermine, there was an excellent correlation between the particle number, the count rate and the diffusion constant [16]. HTAB is known to be a much weaker condensing agent than spermine, hence it follows that they interact differently with DNA. The ability of the compound to condense DNA was routinely evaluated by the concentration required to complete the process (reflected by diminished fluorescence) [17]. Such measurements do not take other factors into account, such as the possible interference of associated dye in the condensation process. The situation is further complicated because the plasmids used do not compose a uniform sample. It is known that two different conformations are present: supercoiled and relaxed. When the cationic compound interacts strongly with DNA (as in the case of spermine), such conformational differences are likely to have a very limited effect on the condensation process. But when a weak condensing compound is used, such DNA conformational differences may become relevant. The presented data may be explained by assuming that the sample consists of two plasmid populations that interact with HTAB differently. The fact that count rate decreases only by half (50%) (Figure 2C) may imply a substantial contribution of free dye to overall fluorescence intensity, and increased fluorescence coming from as yet uncondensed plasmid (the remaining particle number at high HTAB concentrations falls to 15% of its initial value). Differences in the condensation efficiencies of different plasmid populations may explain the observed apparent discrepancy between the particle number and diffusion time dependencies on HTAB concentration. The assumption is that an increase in diffusion time reflects the behavior of only one plasmid fraction, whereas the other condenses progressively with rising cationic compound concentration. When the process is followed over time (Figure 3), particle number and diffusion time remain constant, which validates our conclusion regarding the completeness of plasmid condensation. Only the count rate changes, which shows that dye dissociation takes a long time, and indicates its entrapment within the condensed plasmid-HTAB aggregates. This decrease in count rate is not accompanied by any change in either particle number or diffusion time. Such a result is supported by available data in the literature, indicating that PrIo may remain trapped within collapsed DNA molecules, and that its diffusion is slow.

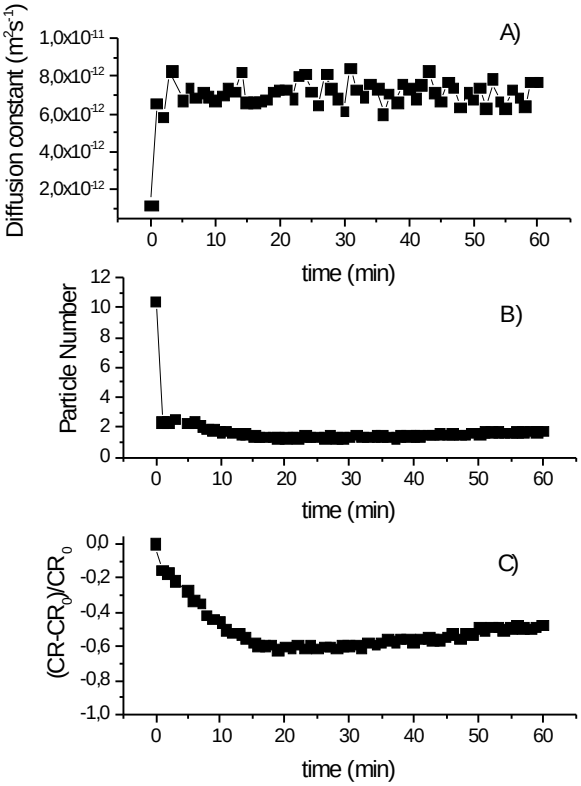

Fig.3. The time evolution of the diffusion time (A), particle number (B), and relative count rate (C) of plasmid labeled with propidium iodide upon the addition of HTAB. 13.3 μM HTAB was added at time zero.

The data presented in this paper show that the FCS technique is a valuable tool in determining the conformational state of DNA molecules. It permits the determination of the cationic compound concentration (here HTAB) needed to induce plasmid condensation and estimate the hydrodynamic size of various plasmid conformational forms. Two additional parameters, count rate and particle number, are difficult to interpret at present. However, the observed apparent inconsistencies indicate the possibility of retrieving additional information about how various compounds interact with DNA molecules. To obtain a complete understanding of the events that lead to cationic compound/DNA aggregate formation (reflected in measured dye fluorescence properties), further studies are required.

CONCLUSION

Synthetic delivery vehicles for genetic material are becoming an important alternative for viral vectors. They do not induce any immunological response and have potentially unlimited capacity for genetic information. Their major disadvantage is their very low transfection efficiency. In order to improve this, a better understanding of the physical chemistry that governs aggregate formation is needed. Complex aggregate structure, with a long DNA molecule and complex amphiphilic ensemble, requires specialized techniques and precise protocols in order to obtain particular desired properties. We present the application of FCS in studying DNA condensation, a process necessary to obtain a well-defined aggregate. DNA molecules were labeled with propidium iodide. When the sample was titrated with HTAB, the count rate, particle number and diffusion time were estimated. The dependence of diffusion time on HTAB concentration shows that a well-defined concentration exists at which a rapid increase in diffusion time is observed. This indicates the condensation process is taking place. The dependence of count rate and particle number on HTAB concentration was different from that observed for Co^{+} or spermine, which means that FCS detects a difference between various condensing agents. The results presented in this paper show that FCS might become a valuable tool in studying the behavior of macromolecules and the supramolecular aggregates they form.

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