LABORATORNÍ PŘÍSTROJE A POSTUPY

COMPACTION MECHANISM OF INTERMEDIATE-SIZED DNA ELUCIDATED BY FLUORESCENCE LIFETIME CORRELATION SPECTROSCOPY

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Introduction

Compaction mechanism of intermediate-sized DNA is not yet understood

In viruses, bacteria, prokaryotes, and sperm cells, DNA is packed into dense structures by positively charged polyamine molecules such as spermine. DNA packaging of single DNA molecules is called DNA compaction and can be generated by a variety of multivalent cations. There is an impressive amount of studies characterizing the morphology of the compacted DNA state by cryoelectron microscopy and it appears to be possible to rationalize the conditions for forming toroides, spherical globules, rods, or rackets[1]. Single molecule fluorescence microscopy allowing to visualize large DNA molecules (166 kbp) clearly demonstrated the discrete ("all-or-non") character of the DNA coil–globule transition[2]. Moreover, Yoshikawa's group could visualize the compaction mechanism by fluorescence microscopy directly in real time[3] and showed that a compact part on a 166 kbp DNA chain appears and grows within several seconds until complete collapse. The same group showed that under certain conditions these intermediate conformations can be stabilized leading to unfolded and compact phases of DNA coexisting along a *single* DNA molecule[1]. It is important to note that all these mechanistic observation based on fluorescence microscopy were done using rather large DNA molecules. For example, for the bacteriophage T4 DNA (166 kbp) that was preferentially used by the above mentioned studies by Yoshikawa, a hydrodynamic radius of about 900 nm and 90 nm was determined for the unfolded and spermine-induced compacted state, respectively[4]. In a recent study[5], fluorescence microscopy together with a Brownian motion analysis was for the first time applied on the spermine-induced compaction of circular plasmide of an intermediate size (12.5 kbp). In contrast to those linear DNA molecules larger than several tens of kbp, the authors found a comparable small change of the hydrodynamic radius from the elongated form (260 nm) to about 150 nm in the compact state and characterized the folding transition as continuous. To our knowledge, this study is the only single-molecule observation of compaction of a non-adsorbed, freely diffusing circular plasmide of an intermediate size. Taking into account the physiological relevance of such „small" DNA structures[5] and the fact that the resolution of the fluorescence microscopy used[5] is less than 230 nm, there is certainly a need for applying alternative techniques which are able to monitor on a single molecule level the spermine-DNA interaction in solution. This motivated us to apply a recently developed single molecule fluorescence technique, fluorescence lifetime correlation spectroscopy (FLCS), for investigating the compaction mechanism of a 10 kbp circular plasmide.

Introduction to Fluorescence Lifetime Correlation Spectroscopy

Fluorescence lifetime correlation spectroscopy (FLCS) has been suggested in 2002 by Böhmer and Enderlein[6] and it was our group that demonstrated its experimental realization[7] and reported its first molecular applications[7,8]. FLCS is based on fluorescence correlation spectroscopy[9,10] (FCS).

FCS is a microscopic technique that monitors fluorescence fluctuations of fluorescently labeled species and characterizes dynamical properties of the fluorophores, such as free diffusion. A laser beam is led to the inverted confocal microscope and by objective with high numerical aperture is focused into a diffraction limited spot. Since the size of the spot is around one femtoliter and provided the concentration of labeled molecules is in the nanomolar range, the number of particles in the observation volume is in the order of units. In this case, the fluctuations in the intensity arise mainly from the diffusion of fluorophores in and out of the focal volume. The fluctuations carry information on the number of particles in the illuminated volume, the mean time the particles spend in the volume (τ_{res}) and consequently, on their concentration and diffusion coefficient. These pieces of information can be obtained by calculation of an intensity autocorrelation function (ACF) and fitting the function to an *a priori* known mathe-

matical model.

This approach works well if there is only one type of diffusing species. For multicomponent analysis, even though mathematical models are available, FCS fails, as there are already too many parameters to be fitted, so the fitting procedure is not mathematically stable. One of the ways that overcomes this problem is so called dual-color FCS,[11] which simultaneously monitors species of different color and allows calculating autocorrelation function for each of them. Apart from that, also crosscorrelation function (CCF) can be obtained, which gives additional information on the interaction between the species of interest, formation of aggregates, for instance. The experimental realization of dual-color FCS usually requires two lasers focused into a single spot (or a laser for two-photon excitation) and two detection paths.

The other, rather new solution of the multicomponent problem is FLCS. Similarly to the dual-color FCS, FLCS distinguishes between fluorophores with different spectroscopic properties. Instead of the emission wavelength used in dual-color FCS, FLCS uses lifetime of fluorescence. The technical realization becomes much easier since only one pulsed laser and a single detection path is needed. FLCS has an intrinsic advantage based on the fact that lifetime, compared to the emission spectra, is much more sensitive to the local microenvironment. Eventually, only a single fluorophore in different locations can be simultaneously monitored.

In this contribution, we take advantage of the finding that the fluorescence lifetime of the DNA intercalating dye PicoGreen® (PG) is shortened upon DNA compaction. The analysis of the point with partially condensed DNA can decide whether the compaction occurs continuously or whether it is a discrete process.

Material and Methods

DNA samples and labeling

The 10 kbp pH βApr-1-Neo plasmid was a gift from the laboratory of Prof. Maciej Ugorski (Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław, Poland). It was prepared as described elsewhere[12] with slight modifications during the final purification stage[13]. The unsymmetrical monomethine cyanine dye PicoGreen® (2-[bis(3-dimethylaminopropyl)amino]-4-(3--methyl-2(3*H*)-benzothiazolylidene)methyl-1-phenyl-quinolinium) was purchased from Molecular Probes (Leiden, Netherlands) and spermine from Sigma. We used the labeling ratio 1 PG/100 basepairs. Experiments were performed in a Tris-EDTA buffer (pH 7.95, 10 mM Tris, 1 mM EDTA) at room temperature (25 °C).

Instrumental setup

FLCS measurements were performed on a MicroTime 200 inverted confocal microscope (PicoQuant, Germany). We used a pulsed diode laser (LDH-P-C-470, 470 nm, PicoQuant) providing 80 ps pulses at 40 MHz repetition rate, dichroic mirror 490 DRLP and band-passfilter 515/50 (Omega Optical), and a water immersion objective (1.2 NA, 60×) (Olympus). Low power of 4 μW (at the back aperture of the objective) was chosen to minimize photobleaching and saturation. In the detection plane, a pinhole (50 μm in diameter) was used and the signal was subsequently splitted on two single photon avalanche diodes (SPADs, PDMs, Microphoton Devises, Bolzano, Italy). For calculating fluorescence correlation curves, we correlated only photons from different SPADs for preventing detector afterpulsing.

Data acquisition and processing

Photon arrival times were stored using fast electronics (Picoharp 300, PicoQuant) in time-tagged time-resolved recording mode. Two independent times were assigned to each detected photon: i) a time after the beginning of the measurement and ii) a time after the previous laser pulse. All the data were acquired for two hours in order to achieve good photon statistics. The FLCS data analysis was done using home built routines (DevC++, Bloodshed Software and OriginPro70, OriginLab Corporation). Further details of the data evaluation are given elsewhere[14].

Results and Discussion

Titration of PicoGreen® (PG)-labeled circular 10 kbp DNA with spermine

When employing the experimental setup for performing FLCS experiments[14], we simultaneously obtain information on the fluorescence lifetime (and thus on the molecule microenvironment) and its diffusion properties

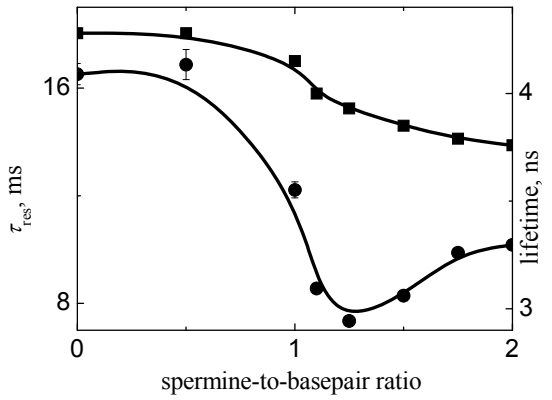

Fig. 1. **Dependence of mean residence time (circles) and fluorescence lifetime (squares) on spermine-to-basepair ratio**

(carried in the fluorescence intensity fluctuations). In Fig. 1, the fluorescence lifetime and the mean residence time τ_{res} of particles in the focus are used for the characterization of the DNA compaction by spermine titration of a PicoGreen® (PG)-labeled circular 10 kbp DNA. For the sake of simplicity, the mean residence time τ_{res} values were obtained by fitting the ACFs to the model assuming a single point-like diffusing species, though being aware that for multiply labeled DNA molecule, the size of which exceeds three times the diameter of the laser focus, segmental motion and the Brownian motion of the whole molecule are superimposed[15,16]. Fluorescence lifetime values were obtained by tail fitting of the time-correlated single photon counting histograms.

Fig. 1 shows that at first, we observe almost no change in τ_{res}. As already reported[4], at these ratios sodium ions in minor grooves[17] are replaced by molecules of spermine. During this exchange, the fluorescence lifetime of PG measured simultaneously does not change either, which means that the dye "sees" the same microenvironment. At the spermine-to-basepair ratio around 1, τ_{res} decreases as well as the lifetime. This moment corresponds to the folding of the DNA molecules that reached the complete neutralization. The lifetime and residence time drops refer to the microenviromental change in close proximity of PG and increase of the diffusion coefficient, respectively. While the lifetime decrease might be caused by functional groups which quench fluorescence getting closer to PG, the increase in the diffusion coefficient is the result of a dramatic decrease of the hydrodynamic radius. When there are enough condensed DNA molecules, simultaneously with folding, the condensed DNA starts to aggregate, which causes prolongation of τ_{res}. The lifetime remains constant as the additional aggregation does not change the immediate microenvironment of PG. From visual inspection of the rather steep residence time dependence, which displays the decrease of the hydrodynamic radius due to compaction induced by spermine, one might speculate that the process is rather discrete than continuous. However, the obtained residence time is an ensemble average over many single molecule diffusion events. Yoshikawa's single molecule observations on large DNA molecules demonstrated that results on DNA-condenser titrations obtained by "ensemble" methods might lead to wrong mechanistic conclusions[1].

FLCS revealing mechanism of DNA compaction

During the FLCS experiment with increasing spermine-to-basepair ratio, fluorescence lifetime of PG undergoes shortening from 4.2 to 3.5 ns. This can be assigned to the formation of folded domains on the DNA chain with different microenvironment of PG. We regard the spermine-to-basepair ratio, when the lifetime of PG equals 3.9 ns, to be the "middle point" of the condensation, where molecules of PG coexist in the unfolded and folded domains of DNA. Using normalized fluorescence decays of the 4-ns (4.2 ns) and 3-ns (3.5 ns) living fluorophores and

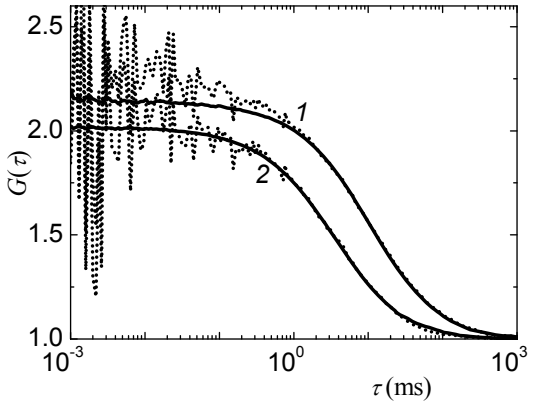

Fig. 2. **Comparison of ACFs measured for free DNA and condensed DNA separately and ACFs obtained from filtering of the titration midpoint.** Solid curves: DNA (curve 1) and condensed DNA (curve 2) measured individually; dotted curves: DNA (curve 1) and condensed DNA (curve 2) obtained from filtering of the titration midpoint

the decay of the compound signal at the middle point, the stasticical lifetime filters can be calculated as described in ref.[14] Applying those filters on the fluctuation photon trace measured at the titration midpoint, the resulting auto- and crosscorrelation functions (ACFs and CCFs, respectively) are expected to be very different for the two considered compaction mechanisms. Following extreme situations can be expected:

a) DNA compaction is a continuous process: all the DNA molecules start to form condensed regions, where the lifetime of PG is shortened, i.e. each DNA molecule contains both 4-ns and 3-ns lifetime fluorophores. In this case, the residence time obtained for the 4-ns component is supposed to be shorter compared to the residence time of the free DNA measured separately. And vice versa, the residence time of the 3-ns component is prolonged in comparison with condensed DNA separately measured. Since the coexistence of both dye types on a single molecule is expected, significant crosscorrelation appears.
b) DNA compaction is a discrete "all-or-none" process: DNA molecules coexist in the uncondensed and in the condensed form. In this case, the ACF (and consequently the residence time) of the 4-ns and 3-ns component exactly matches the ACF of separately measured DNA and condensed DNA, respectively. All the DNA molecules are either in the unfolded, or in the folded state. Thus, no crosscorrelation is expected.

Comparison of the ACFs depicted in the Fig. 2 clearly shows that the latter case is the correct one, i.e. the compaction is a discrete transition. The non-one CCFs suggest, however, that apart from the majority of the molecules that are in one of the states, there is a fraction of molecules bearing both the 3-ns and 4-ns lifetime components. Close

inspection of the amplitudes of the CCFs even suggests that there is an equilibrium state dynamics between compact and free domains on the DNA chain[16].

Conclusions

The compaction mechanism of intermediate-sized DNA can neither be revealed by ensemble techniques nor by fluorescence microscopy imaging. FLCS opens the possibility to characterize the conformation of DNA molecules at the midpoint of the spermine titration. In opposite to a statistical analysis of the Brownian motion of 12.5 kbp DNA molecules[15], FLCS clearly shows that the spermine induced compaction of a 10 kbp circular plasmide occurs in the all-or-non regime. There is only a small fraction of molecules (not observable in ACFs) that contain both the 3-ns and 4-ns labeled domains. These molecules are responsible for the CCFs curves, suggesting not only a static coexistence of the domains but an equilibrium interchange between the folded and unfolded ones. Thus, we show for the first time that the general mechanism of DNA compaction is similar for intermediate-sized and large DNA molecules.

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Compaction Mechanism of Intermediate-sized DNA Elucidated by Fluorescence Lifetime Correlation Spectroscopy

This work reveals the compaction mechanism of intermediate-sized DNA molecules. Using the FLCS it was shown that the compaction of 10 kbp circular plasmide is the all-or-none transition.