Eur Biophys J (2001) 30: 98–109
DOI 10.1007/s002490000129

ARTICLE

M. L. Barcellona · Y. Chen · J. D. Müller · E. Gratton

4′,6-Diamidino-2-phenylindole (DAPI) interacts with rare structures of GC polymers

Received: 25 July 2000 / Revised version: 30 November 2000 / Accepted: 30 November 2000 / Published online: 29 March 2001

Abstract The binding of 4′,6-diamidino-2-phenylindole (DAPI) to double-stranded GC polymers either in the alternating or in homopolymer sequence was investigated using fluorescence techniques. We employed fluctuation correlation spectroscopy, which measures the diffusion coefficient of fluorescent particles, to demonstrate that the fluorescence was originating from relatively slowly diffusing entities. These entities display a very large heterogeneity of diffusing coefficients, indicating that molecular aggregation is extensive in our samples. We used frequency domain fluorometry to characterize the fluorescence lifetime of the species, while varying the GC polymer-dye coverage systematically. At very low coverage we observed a relatively bright fluorescent component with a lifetime value of approximately 4 ns. The stoichiometry of binding of this bright species was such that it can only arise from rare molecular structures, either unusual loops or large molecular aggregates. The amount and characteristics of this bright fluorescent component were different between the homo and the alternating polymer, indicating that the difference in sequence of the two polymers is responsible for the different aggregates which are then detected in the fluorescence experiment. At large GC polymer coverage we observed a relatively wide distribution of fluorescent species with short lifetime values, in the range between 0.12 and 0.2 ns. Given the stoichiometry of binding of this fluorescent component, we concluded that it could arise either from intercalative and/or non-specific binding to the DNA double-stranded molecules. We comment on the origin of the rare but brightly fluorescent binding sites and discuss the potential to detect such unusual DNA structures.

Keywords Fluorescence correlation spectroscopy · Aggregation · Fluorescence lifetime · DAPI/DNA interactions

M. L. Barcellona (✉)
Department of Biochemistry and Molecular Biology,
Universty of Catania, Città Universitaria,
Viale A. Doria 6, 95125 Catania, Italy
E-mail: marsirin@mbox.unict.it
Tel.: +39-095-7384064/330829
Fax: +39-095-7384222

Y. Chen · J. D. Müller · E. Gratton
Laboratory for Fluorescence Dynamics,
University of Illinois at Urbana-Champaign,
Urbana, Illinois, USA

Introduction

The interaction of 4′,6-diamidino-2-phenylindole (DAPI) with DNA and polydeoxynucleotides has been extensively studied since the drug was synthesized by Dann et al. (1971). DAPI exhibits potent cytotoxic activity against some parasites and this biological effect is commonly attributed to its capacity to bind to DNA and to interfere with DNA metabolism. Many efforts have been devoted to unravel the DAPI binding mechanism to nucleic acids. It is now generally accepted that DAPI binds to DNA preferentially at AT-rich regions within the minor grove of B-DNA in solution, forming highly fluorescent complexes. Time resolved fluorescence studies reveal a biexponential decay behavior for DAPI in buffered solutions (0.18 ns and 2.5 ns). When bound to AT-containing polymers, DAPI has a single lifetime component of about 4 ns at all DNA-dye coverage ratios. It has also been reported that DAPI can interfere with enzyme activity related to nucleic acid metabolism. For example, a strong inhibitory effect upon RNA polymerase II activity was observed, which is attributed to the high affinity binding of DAPI to AT sequences in the minor groove (Chiang et al. 1994). It is known that the promoter region includes a six base pair sequence located upstream of the initiation site in the 5′ direction. Such a sequence, called the ''Pribnow box'' or ''TATA box'', can be made silent in the presence of DAPI because of its interference with the binding of TBP (TATA-binding protein) to its consensus sequence, preventing formation of the transcription factors-DNA

complex which is required by the RNA polymerase II for initiating gene transcription. Other enzymes, such as the exonuclease III, DNA polymerase I, and DNA ligase, can be actively inhibited by DAPI interaction with AT sequences, acting as enzyme substrates (Palù et al. 1987; Straney and Crothers 1987; Parolin et al. 1995). However, the complex heterogeneity of functions displayed by DAPI cannot be attributed to the binding to AT sequences alone. In fact, a number of studies have shown that the fluorescence characteristics of DAPI depend on DNA sequence and that DAPI can interact with GC sequences, although with less affinity than to AT sequences. So far, the molecular mechanism responsible for the interaction between DAPI and GC sequences has not been properly clarified (Wilson et al. 1990; Tanious et al. 1992; Eriksson et al. 1993; Kim et al. 1993; Sehlstedt et al. 1993; Tanious et al. 1994; Colson et al. 1995). Intercalation of the dye between GC or mixed AT/GC sites was originally proposed by Wilson et al. (1989, 1990). The reduced depth and increased width of the minor groove of GC compared to AT sequences could lead to DAPI intercalation.

Whatever is the modality of binding of this drug to GC sequences, either intercalation or major grove insertion (Kim et al. 1993), the protruding 2-amino group of guanine, hindering the access to the floor of the minor groove, could represent a critical element in the drug-DNA recognition process preventing DAPI from binding to GC base pairs in the minor groove. This steric hindrance could be a limiting factor for a less favorable minor groove binding of DAPI to GC-containing polynucleotides. While these qualitative arguments are appealing, there exists no consensus regarding the nature of the DAPI-GC interaction.

While the minor-groove binding modes of several fluorescent divalent cations with DNA containing AT bases have been studied extensively, substantially less information is available on similar complexes with DNA sequences containing GC bases. Some special functional regions of chromosomal DNA, such as the telomeres, are represented by short, repetitive sequences of GGG, repeated hundred of times. Although there is a very clear demonstration of the preferential binding of DAPI to AT sequences, the lack of DAPI affinity to GC sequences has been attributed either to the steric hindrance exerted in the minor groove by the exocyclic amino group of guanine or to the decreased attraction of the electronegative potential.

In this work we used different experimental approaches to address the original question: does DAPI bind to GC-containing polymers and how? It is known that DAPI interacts predominantly between homopolymer duplexes with respect to the alternating polymer in two different orientations differing by a 180° flip about its short axis. These orientations are defined according to the phenyl ring of DAPI facing towards the 5′ end or 3′ end, respectively, of the pyrimidine strand in a homopolymer duplex. This mode of binding does not occur in oligodeoxynucleotides possessing A and T or G and C bases, because of intrinsic symmetry along the two directions of the DNA molecule for these polymers. The relevance for considering both these orientations was supported by various experimental observations (Patel and Canuel 1978; Klevit et al. 1986; Coll et al. 1987, 1989). For this reason, in our experiments we used the two structural classes of GC polymers.

We used frequency domain fluorometry for the investigation of the dynamic fluorescence properties of DAPI, complexed with polyGC polymers in homopolymeric and in alternating sequences. The complexes exhibited multiexponential decay of fluorescence. The populations of the decay components did not follow any single specific binding ratio between DAPI and the base concentration of the different GC polymers, inducing us to postulate a variety of interactions in which higher molecular organization, rather than base composition and sequences, was involved in the binding of DAPI to the GC polymers. Although the two structural classes of polymer behave differently, we were unable to identify specific spectroscopic components with different modes of interaction of DAPI with the CG polymers. To prove that binding occurs, we used two-photon fluorescence correlation spectroscopy (FCS), which is able to distinguish the DAPI probe bound to the polymer from the probe freely diffusing in solution. FCS is a technique in which temporal fluctuations in the fluorescence emitted by the sample are analyzed to obtain information about the processes causing the fluctuations. The principles of FCS were developed more than 20 years ago (Elson and Madge 1974; Madge et al. 1974; Aragon and Pecora 1976; Weissman et al. 1976). Technical improvements made over the last few years have made FCS a relatively straightforward technique applicable for various macromolecular systems having suitable fluorescent indicators (Eigen and Rigler 1994; Berland et al. 1995; Kinjo and Rigler 1995; Berland et al. 1996; Klinger and Friedrich 1997; Maiti et al. 1997; Schwille et al. 1997; Wennmalm et al. 1997; Björling et al. 1998; Kinjo et al. 1998; Rigler et al. 1998; Widengren and Rigler 1998; Chen et al. 1999; Winkler et al. 1999; Wohland et al. 1999; Rippe 2000). FCS measurements allow the characterization of kinetic and thermodynamic processes such as molecular diffusion from the temporal intensity autocorrelation function. The FCS experiments clearly demonstrate that the polymers were highly aggregated and that binding occurred to supramolecular complexes. These complex aggregates may be different for the homo and alternating polymer, providing us with a better insight on the origin of the fluorescent components.

Materials and methods

Polyd(G)·polyd(C), polyd(GC), polyd(A)·polyd(T), and polyd(AT) were obtained from Amersham Pharmacia Biotech. The average molecular weights of the double-stranded polyd(G)·polyd(C), formed by the two homopolymers polydeoxyguanylic acid and polydeoxycytidilic acid, and of the alternating copolymers, polyd(GC), were about 5.5×10^6 and 4.45×10^6, respectively, with

an approximate average length in base pairs of 8560 and of 7000, respectively. To maintain a double-stranded configuration in solution, the polymers were rehydrated with 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl (buffer A). Phosphate buffer choice was motivated by its pH stability to temperature variation (Stoll and Blanchard 1990).

To assess the homogeneity of the molecular weight and retention of double-stranded conformation in polymer samples, agarose gel electrophoresis (0.7% w/v) was performed in Tris-phosphate buffer (50 mM) in the presence of 0.5 μg/mL of ethidium bromide.

When large molecular aggregates were present, either they did not migrate into the gel or a significant smear was observed. This behavior arises from differences in size and flexibility of the two classes of polymer and in variable polydispersity from one batch to another for a given polynucleotide. The absorption ratio A_{260}/A_{280} was 1.9, providing an estimation of polynucleotide purity.

DAPI dihydrochloride was obtained from Boehringer Mannheim and checked for purity by thin layer chromatography. All measurements, unless otherwise stated, were performed in the following buffer: 0.1 M phosphate buffer, 0.1 M NaCl, and 0.01 M EDTA (pH 7.4). All the reagents were of the highest purity available and doubly distilled water, Millipore filtered, was used. The concentration of the solutions was determined by using the following molar extinction coefficients at 253 nm for the polyd(G)·polyd(C) (7400), at 254 nm for the polyd(GC) (8400), at 260 nm for the poly(dA)·poly(dT) (6000), and at 262 nm for the polyd(AT) (6600) (Grant et al. 1968; Wells et al. 1970). A molar extinction coefficient of 23,000 M^{-1} cm^{-1} at 342 nm was used to determine the concentration of DAPI solution.

The nominal concentration of the GC polymers, used for FCS experiments, was below 1 μM (base) which, given the average length of the polymers, corresponded to a concentration of less than 50 pM. Since the characteristic two-photon excitation volume of our microscope is about 0.1 fL, this concentration corresponds to about 0.003 polymer molecules in the volume at any instant of time.

Depending on the final concentration of the respective polymer, different ratios of polymer, as moles of phosphate (P) to DAPI (D), were used. We kept the polymer molarity constant, but varied the DAPI concentration. All measurements were carried out at room temperature.

Steady-state spectrofluorometric spectra of DAPI in buffer solution and complexed with AT and GC polymers were recorded with the microprocessor-controlled photon counting spectrofluorometer (ISS, Champaign, Ill.) at 470 nm and 340 nm, for the emission and the excitation wavelength, respectively. Lifetime measurements were performed by the multifrequency phase and modulation fluorometer, described by Gratton and Limkeman (1983), equipped with an ISS1ADC interface for data acquisition and analysis. The excitation source was a continuous wave helium-cadmium laser (Liconix), emitting at 325 nm. In each experiment a set of 10–12 different modulation frequencies were employed in the range between 10 and 250 MHz. The emission was observed using a RG 370 band-pass filter (Janos Technology). A solution of POPOP in ethyl alcohol was used as a reference with a lifetime value of 1.35 ns. The concentration of polymer used in lifetime measurement is $\sim 10^{-3}$ M phosphate. We kept the polymer molarity constant, but varied the DAPI concentration.

Phase and modulation data were analyzed either using a sum of exponentials (Gratton et al. 1984), or by a continuous distribution of lifetime values of Lorentzian shape, which appears to be more appropriate for the analysis of the decay of these complexes (Alcala et al. 1987), where different binding situations occur owing to the high structural variability of GC polymers in both sequences.

In some measurements, pertaining to FCS studies, the two classes of GC polymers were heated for about 15 min to the melting transition region, at 102 °C for the alternating copolymers and 95 °C for the homopolymers; stacking energy: 9.7 and 8.2 kcal/mol per stacked pair, respectively (Ornstein et al. 1978). The two stages of the denaturation process were followed by the increase in the absorbed light at 260 nm. Subsequently, the samples were slowly cooled in an ice-water bath, by favoring interstrand nucleation. This event, being a rate-limiting step in the renaturation process, lasted between 15 and 20 min. The cooled samples were equilibrated at room temperature for 10 min, before measuring with the FCS apparatus.

FCS instrumentation

The instrumentation for two-photon fluctuation correlation experiments is similar to that described by Berland et al. (1995). The experiments were carried out using a Zeiss Axiovert 135 TV microscope (Thornwood, NY) with a 40× Fluar oil immersion objective (NA = 1.3). A mode-locked Ti:sapphire laser (Mira 900, Coherent, Palo Alto, Calif.) pumped by an Innova 410 argon ion laser (Coherent) was used as the two-photon excitation source. For all measurements, an excitation wavelength of 780 nm was used, and the average power at the sample ranged from 7 to 20 mW. The excitation volume was calibrated by fluorescein in high pH buffer [50 mM Tris (hydroxymethyl) aminomethane (Tris) buffer (Sigma, Mo.), pH 9.5] by using the diffusion coefficient of fluorescein at a value of 300 $\mu m^2/s$ (Chen et al. 1999). The optical table is supported by a pneumatic isolation system to reduce mechanical vibrations. Photon counts were detected by an APD (EG&G, SPCM-AQ-141). The output of the APD unit, which produces TTL pulses, was directly connected to the data acquisition card. The photon counts were sampled at 20 kHz. The recorded and stored photon counts were later analyzed with LFD Globals Unlimited software (Champaign, Ill.) and programs written for PV-WAVE version 6.10 (Visual Numerics).

FCS formalism

Numerous publications dealing with the basic theory of FCS have appeared (Elson and Madge 1974; Thompson 1991; Maiti et al. 1997; Schwille et al. 1997; Widengren and Rigler 1998; Meseth et al. 1999). FCS measures diffusion times and concentrations by evaluating time-resolved measurements of fluctuations in the molecular fluorescence emission that have their origin in the Brownian motion of the fluorophores through a small volume ($\sim 10^{-15}$ L). This volume element is created by focusing the excitation light beam to a diffraction-limited spot using a high numerical aperture objective. The measured fluctuations in the fluorescence intensity $F(t)$ depend on the diffusion of molecules in and out of the volume. Such fluctuations $[\delta F(t)]$, in the measured fluorescence $F(t)$ from the average fluorescence $\langle F \rangle$, are then analyzed by their autocorrelation function, $G(\tau) = \langle F(t) \cdot F(t+\tau) \rangle / \langle F(t) \rangle^2 - 1$, where $\langle\ \rangle$ indicates time average operation and τ is the correlation time. Typical values of the beam waist in our two-photon microscope are 300 nm for the radial direction and 800 nm for the axial direction.

The measured autocorrelation function is compared to a theoretical one derived for a particle diffusing freely through an excitation volume. The value of the autocorrelation function extrapolated at $\tau = 0$, the $G(0)$ value, characterizes the amplitude of the intensity fluctuations. For a single diffusing species, $G(0)$ is inversely proportional to the average number of particles $\langle N \rangle$ in the excitation volume, i.e. $G(0) \propto 1/\langle N \rangle$. For a sample containing several species diffusing in a volume element, which is characterized by a three-dimensional Gaussian shape, the autocorrelation function is given by:

$$G_{\mathrm{tot}}(\tau) = \sum_{i=1}^{M} f_i^2 \, G_i(0) \left(1 + 8\frac{D_i \tau}{w_0^2}\right)^{-1} \left(1 + 8\frac{D_i \tau}{w_z^2}\right)^{-1/2} \qquad (1)$$

where $G_{\mathrm{tot}}(\tau)$ is the global autocorrelation function, given as a weighted sum of the autocorrelation function of each species i. The weighting factor is given by the square of the fractional intensity $f_i = F_i / F_{\mathrm{tot}}$, where F_{tot} is the total fluorescence intensity and F_i is the intensity associated with species i. Each individual autocorrelation function depends on its corresponding $G_i(0)$ value and its diffusion coefficient, D_i. The beam waist in radial and axial direction is given by w_0 and w_z, respectively.

Results

Steady-state measurements

Irrespective of the base content and sequence, the emission spectrum of DAPI bound to different polydeoxynucleotides at high and intermediate P/D ratios showed a blue shift of the emission maximum by 15 nm for alternating GC complexes, by 10 nm for AT complexes, and by 5 nm for homopolymeric GC complexes, with respect to the emission maximum of free DAPI in buffered solution (470 nm) (Figs. 1 and 2). The complexes with AT polymers, in both sequences, exhibited the same spectroscopic pattern at all P/D ratios examined (Barcellona and Gratton 1989, 1990). In contrast, the complexes formed between DAPI and GC polymers differ in their spectroscopic behavior at low P/D ratios and in addition on the GC sequences (Figs. 1 and 2). For polyd(GC)/DAPI complexes at P/D ratios ranging from 10 to 1, the maximum emission wavelength was blue shifted (455 nm) compared to that of free DAPI. At the same P/D ratios, the complexes formed between DAPI and GC polymers in the homopolymeric sequences showed a red shift with decreasing P/D ratio until $P/D = 1$, when the emission maximum was close to that of free DAPI. The full width at half maximum (FWHM) was also changed: for free DAPI the FWHM is 113 ± 2 nm, whereas that of DAPI bound to polyd(GC) is 87 ± 2 nm, and in the same order of magnitude was that of DAPI bound to poly(dG)·poly(dC). The shapes of the emission spectra were almost independent of the P/D ratio and of the excitation wavelength.

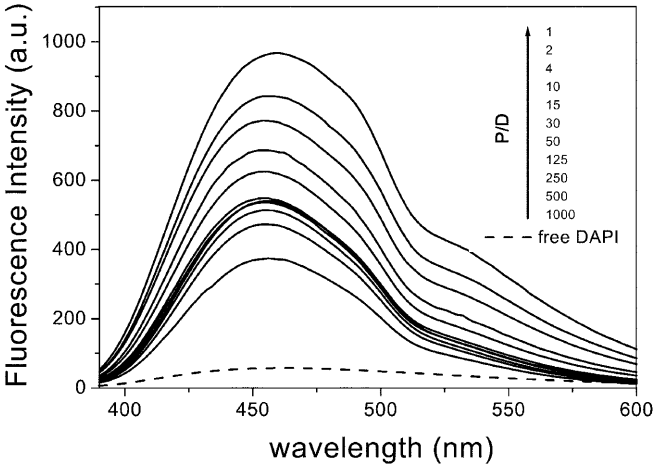

Fig. 1 Emission spectra of free DAPI in buffered solution (*dashed line*) and bound to poly(dG)·poly(dC) at different P/D ratios from 1000 (*bottom*) to 1 (*top*). The excitation wavelength was 342 nm

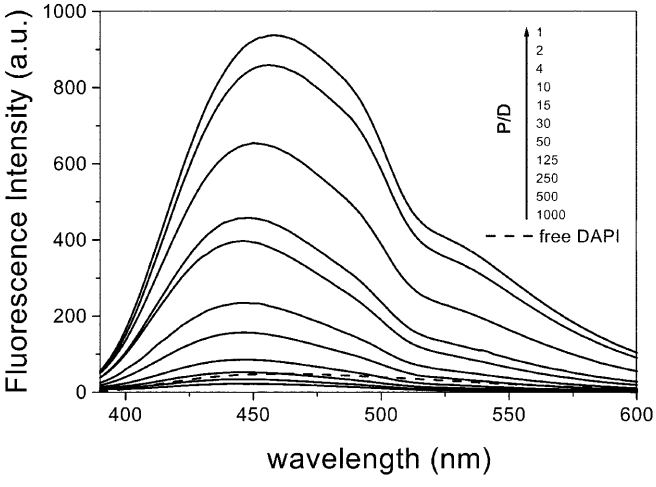

Fig. 2 Emission spectra of free DAPI in buffered solution (*dashed line*) and bound to polyd(GC) at different P/D ratios from 1000 (*bottom*) to 1 (*top*). The excitation wavelength was 342 nm

A significant fluorescence increase for poly(dG)·poly(dC)/DAPI complexes was observed at all P/D ratios examined. In accordance with other literature reports (Manzini et al. 1983, 1985; Szabo et al. 1986; Barcellona and Gratton 1989, 1990), no "apparent" fluorescence increase was observed for polyd(GC)/DAPI complexes at high P/D ratios (from 1000 to about 250), but rather there was a 40% decrease in the fluorescence intensity, becoming equal to that of free DAPI in solution at a P/D ratio of 250 with a maximum emission at 462 nm (Fig. 2). It has been previously reported that the lack of fluorescence enhancement could be ascribed to a loosely fitting binding and/or to a less hydrophobic environment experienced by the drug, although the various spectral changes of the emission spectrum, together with previous circular dichroism measurements (Manzini et al. 1983), were indicative of an interaction between the dye and the polymer (Barcellona and Gratton 1990). Since the less fluorescent species in DAPI decay comes from an intramolecular proton transfer in the excited state, the decreased fluorescence intensity of DAPI upon binding could result from a change in the DAPI solvation, affecting the proton transfer efficiency in the excited state (Barcellona and Gratton 1990; Kim et al. 1993).

Lifetime measurements

The fluorescence decays of DAPI complexed with GC polymers in homopolymeric and in alternating sequences, at different P/D ratios from 1000 to 1, were measured at modulation frequencies in the range from 10 to 250 MHz (Tables 1 and 2). Phase and modulation values obtained with poly(dG)·poly(dC)/DAPI complexes at high P/D ratios (from 1000 to about 50) were analyzed with a sum of exponential components and by a continuous distribution of lifetimes of Lorentzian shape. Both models gave satisfactory fits. The two-exponential fit gave components with lifetimes of 3.9 ns and 0.18 ns. The χ^2 value reduces slightly with the distributional analysis. The fractional fluorescence intensities of the long lifetime component were between

Table 1 Fluorescence decay parameters for the poly(dG)·poly(dC)/DAPI complexes at different P/D ratios. P/D = moles of phosphate (P) to DAPI (D); τ_1 = lifetime of the long decay component in nanoseconds; τ_2 = lifetime of the short decay component in nanoseconds; f_1 = fractional fluorescence intensity of the long decay component; W_1 and W_2 = widths in nanoseconds, as FWHM, of the long and the short decay components, respectively; and χ^2 = the reduced χ^2

Complex	P/D	Double exponential		f_1	χ^2	Lorentzian distribution	
		τ_1	τ_2			W_1	W_2
Poly(dG)·	1000	3.91	0.20	0.93	0.86	0.01	0.31
poly(dC)/D	500	3.82	0.19	0.91	1.83	0.01	0.43
	250	3.80	0.18	0.89	1.65	0.01	0.51
	125	3.78	0.17	0.86	0.91	0.08	0.41
	50	3.67	0.15	0.76	1.52	0.02	0.56
	30	3.46	0.15	0.69	1.04	0.01	0.51
	15	3.25	0.12	0.64	0.96	0.01	0.31
	10	3.01	0.12	0.55	0.33	0.12	0.45
	4	2.92	0.12	0.49	0.57	0.13	0.21
	2	2.89	0.11	0.43	1.14	0.07	0.21
	1	2.88	0.11	0.38	0.91	0.06	0.23
Poly(dA) ·	1000	3.9	–	1.00	0.52	0.10	–
poly(dT)/D	10	3.9	–	1.00	0.42	0.10	–
	1	3.9	0.20	0.50	0.38	0.10	0.10
DAPI	–	2.8	0.18	0.35	0.92	0.30	0.15

Table 2 Fluorescence decay parameters for the polyd$(GC)_2$/DAPI complexes at different P/D ratios. P/D = moles of phosphate (P) to DAPI (D); τ_1 = lifetime of the long decay component in nanoseconds; τ_2 = lifetime of the short decay component in nanoseconds; f_1 = fractional fluorescence intensity of the long decay component; W_1 and W_2 = widths in nanoseconds, as FWHM, of the long and the short decay components, respectively; and χ^2 = the reduced χ^2

Complex	P/D	Double exponential		f_1	χ^2	Lorentzian distribution	
		τ_1	τ_2			W_1	W_2
Polyd$(GC)_2$/D	1000	3.93	0.21	0.45	1.10	0.18	0.78
	500	3.35	0.19	0.43	0.97	0.45	0.42
	250	3.10	0.18	0.44	1.10	0.44	0.51
	125	2.86	0.18	0.43	1.03	0.54	0.47
	50	2.82	0.17	0.43	1.13	0.01	0.31
	30	2.82	0.15	0.39	1.01	0.01	0.36
	15	2.86	0.15	0.36	1.51	0.12	0.16
	10	2.75	0.14	0.36	0.77	0.14	0.35
	4	2.73	0.12	0.34	1.12	0.17	0.12
	2	2.64	0.11	0.35	0.61	0.09	0.06
	1	2.59	0.12	0.34	0.34	0.08	0.03
Polyd$(AT)_2$/D	1000	3.9	–	1.00	0.48	0.10	–
	10	3.9	–	1.00	0.51	0.10	–
	1	3.9	0.20	0.50	0.42	0.10	0.10
DAPI		2.8	0.18	0.35	0.92	0.30	0.15

0.95 ($P/D = 1000$) and 0.75 ($P/D = 50$). By decreasing the P/D ratio to 4, the long lifetime component decreased gradually and constantly (from 3.9 to 2.9 ns) while the relative fractional fluorescence intensity was reduced to 0.49 and, at a P/D ratio of 1, to about 0.38.

For the complexes between DAPI and GC polymers in alternating sequence, the long lifetime component at high P/D ratio (1000) showed the same lifetime value as the companion complex, but did not exhibit the same pattern as the P/D ratio decreased. A drop of the long lifetime to 2.86 ns at a P/D ratio of 125 was observed. The fractional fluorescence intensities were between 0.45 and 0.55 for the long and the short decay component, respectively, at almost all the P/D ratios examined. By progressively lowering the P/D ratio to 1, the long lifetime component decreased to 2.6 ns, with a fractional fluorescence intensity of about 0.34.

For the complexes formed by the homopolymeric GC sequences, the width of the Lorentzian distribution associated with the long lifetime component was very narrow at all the P/D examined, while it was broader for the alternating GC sequences ($W_1 = 0.2$ ns ($\bar{x}$)). The distribution analysis for the short decay components showed a larger distribution width, indicating a significant heterogeneity of binding situations. For the polyd(GC)/DAPI complexes the width was 0.5 ns and for the poly(dG)·poly(dC)/DAPI complexes it was 0.3 ns. The center of the distribution of the short lifetime component was independent of the P/D ratio and of the GC polymer sequence.

For comparison with FCS experimental conditions we performed some lifetime measurements after the same heat treatment procedure (data not shown), at high and intermediate P/D ratios (1000 and 15). The two classes of GC polymers exhibited a similar behavior: they maintained the same bi-exponential decay pattern as before the heat treatment at both P/D ratios examined. However, in the alternating GC polymers there was a slight increase of the fractional fluorescence intensities associated with the long decay components for both P/D ratios (from ~0.45 to ~0.60, at the P/D ratio of 1000 and from ~0.35 to 0.50 at the P/D ratio of 15). The long lifetime component decreased from 3.8 ns to 2.7 ns at the P/D ratio of 1000 and from 2.85 to 2 at the P/D ratio of 15. This result further convinced us that the long lifetime components were due to particular structural motifs that were partially different for the homopolymer and the alternating GC polymers. Since these polynucleotides have unusual self-assembling properties, it is likely that larger molecular aggregates and/or particular molecular organizations could be responsible for the long lifetime component. In the next section we address the problem of molecular aggregation using FCS.

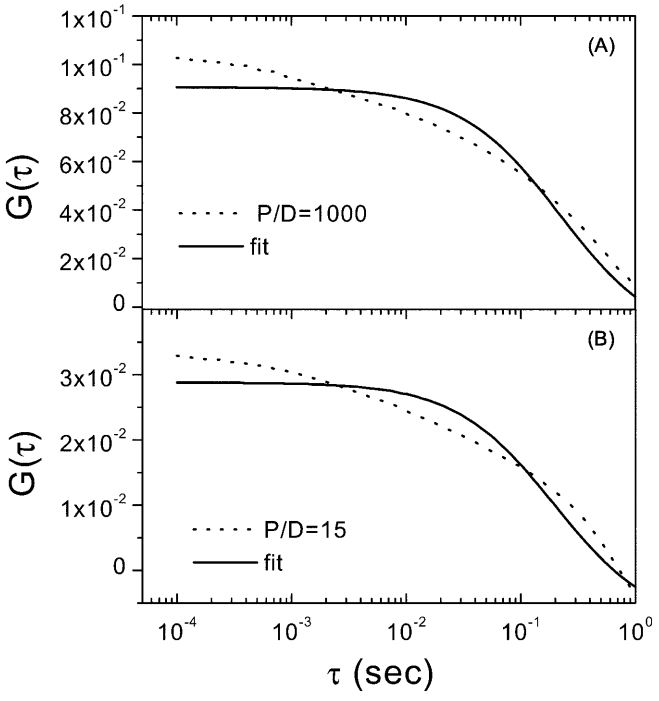

Fig. 3 Autocorrelation function of poly(dG)·poly(dC)/DAPI complexes at a P/D ratio of 1000 (**A**) and 15 (**B**). The *dashed line* represents the experimental autocorrelation function and the *solid line* shows the fit using a single species model. The resulted diffusion coefficients and $G(0)$ values are shown in Table 3

FCS measurements

The autocorrelation function of free DAPI could not be measured under our experimental conditions. The molecular brightness of the free dye is not sufficient to acquire the necessary statistics within reasonable data acquisition times. Based on its molecular size, DAPI will diffuse with a diffusion coefficient similar to the dye fluorescein. Once DAPI is bound to DNA the diffusion coefficient decreases drastically owing to the enormous molecular mass increase of the diffusing entity upon binding. The autocorrelation function of the DAPI-DNA complexes could be measured directly (see Figs. 3, 4, 5, 6). Free DAPI, because of its low molecular brightness, does not contribute to the autocorrelation trace. However, free DAPI adds background intensity to the measurement and leads to a reduction of the amplitude of the autocorrelation signal without affecting the shape of the autocorrelation function.

We measured the autocorrelation function of DAPI bound to homo-GC (Fig. 3), alternating GC (Fig. 5), and alternating AT polymers (the homopolymeric sequence had the same behavior) (Fig. 6) at two different P/D ratios (1000 and 15). All autocorrelation functions were fit to a single species model. The fit parameters are compiled in Table 3. We observed an extremely broad decay curve of the autocorrelation function of the homo-GC sample compared to that of a single species (Fig. 3), which indicates a broad distribution of diffusion times. A distribution of heteropolymer aggregates is consistent with a broader decay curve. Resolution of the heterogeneity of the polymer aggregates by the autocorrelation function depends on many model-dependent parameters. Thus, we restrict ourselves to a more

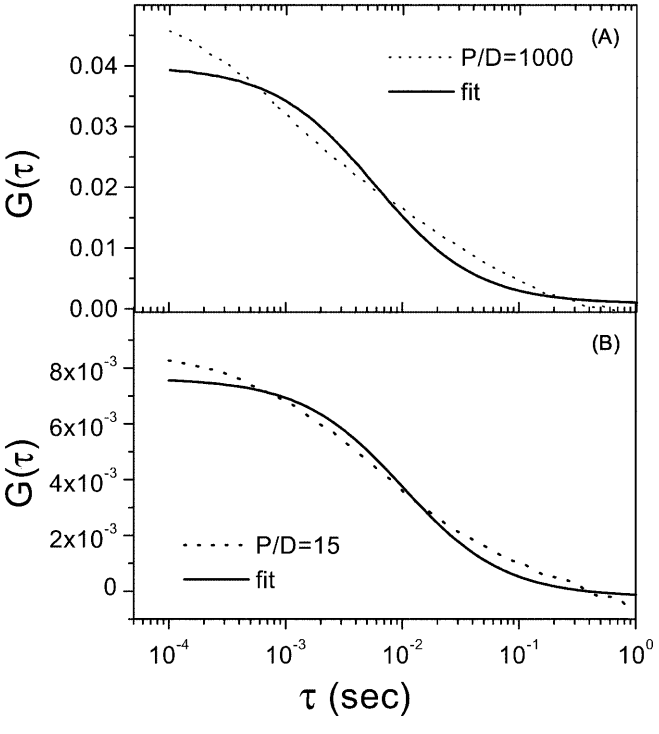

Fig. 4 Autocorrelation function of poly(dG)·poly(dC)/DAPI complexes at a P/D ratio of 1000 (**A**) and 15 (**B**) after heat treatment (see Materials and methods). The *dashed line* is the experimental autocorrelation function and the *solid line* shows the fit using a single species model

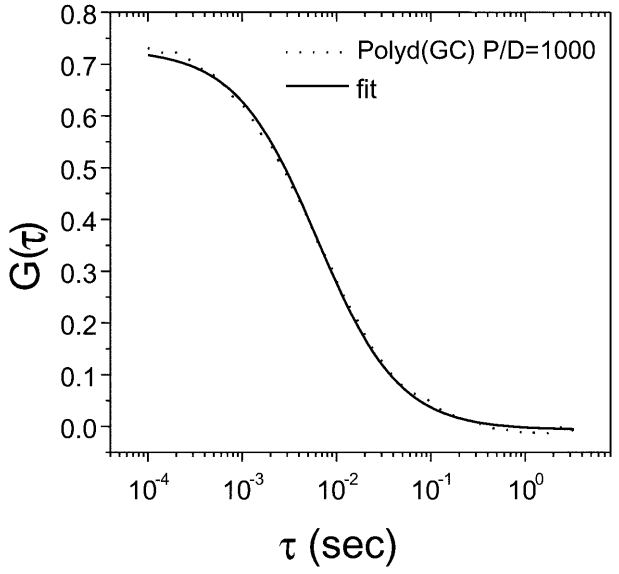

Fig. 5 Autocorrelation function of polyd(GC)/DAPI complex at a P/D ratio of 1000. The *dashed line* is the experimental autocorrelation function and the *solid line* is the fit using a single species model

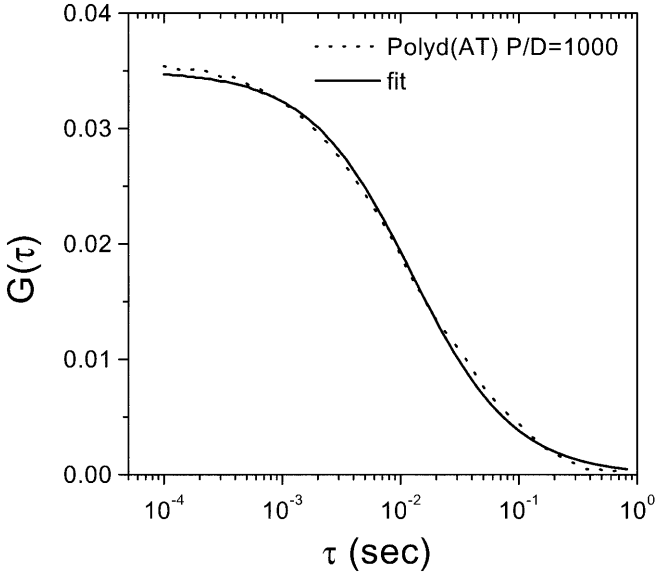

Fig. 6 Autocorrelation function of polyd(AT)/DAPI complex at a P/D ratio of 1000. The *dashed line* is the experimental autocorrelation function after the heat treatment and the *solid line* represents the fit using a single species model

qualitative discussion of the autocorrelation function. We define a correlation time of the autocorrelation function by the time at which the amplitude of the function decayed to half of its original value. The homo-GC polymer exhibits a correlation time of 0.1 s independent of the P/D ratio. Decreasing the P/D ratio from 1000 to 15 by increasing the dye concentration, while keeping the polymer concentration constant, leads to essentially the same shape of the autocorrelation function.

Table 3 Comparison of diffusion coefficient, D and fluctuation amplitude, G(0) before and after the heat treatment for GC and AT/DAPI complexes. The diffusion coefficients and $G(0)$ values of the different polymer complexes are recovered by fitting the autocorrelation function to a single species model with Eq. (1)

	Poly(dG)·poly(dC)/D, $P/D = 1000$	Poly(dG)·poly(dC)/D, $P/D = 15$	Polyd(GC)/D, $P/D = 1000$	Polyd(GC)/D, $P/D = 15$	Polyd(AT)/D, $P/D = 1000$
Before heating, $G(0)$	0.12 ± 0.01	0.04 ± 0.004	0.72 ± 0.072	0.07 ± 0.007	0.16 ± 0.01
Before heating, D (cm^2/sec)	$3.65\times10^{-10} \pm 3.6\times10^{-11}$	$3.81\times10^{-10} \pm 3.8\times10^{-11}$	$1.20\times10^{-8} \pm 1.2\times10^{-9}$	$1.5\times10^{-8} \pm 1.5\times10^{-9}$	$1\times10^{-9} \pm 1\times10^{-10}$
After heating, $G(0)$	0.05 ± 0.005	0.01 ± 0.001	0.42 ± 0.04	0.02 ± 0.002	0.04 ± 0.004
After heating, D (cm^2/sec)	$1.4\times10^{-8} \pm 1.4\times10^{-9}$	$3.5\times10^{-9} \pm 3.5\times10^{-10}$	$1.6\times10^{-8} \pm 1.6\times10^{-9}$	$0.9\times10^{-8} \pm 0.9\times10^{-9}$	$6.01\times10^{-9} \pm 6\times10^{-10}$

The reaction kinetics of DAPI binding to the polymer is visible in the autocorrelation curve, if this rate is faster than the diffusion time scale (Elson and Madge 1974). Binding of DAPI to the DNA is a bimolecular reaction and the reaction rate depends on the dye concentration. The shape of the autocorrelation function, on the other hand, does not change, while varying the dye concentration by almost two orders of magnitude. Thus, the DAPI binding rate has to be slower than about 0.5 s. In other words, the binding sites of the homopolymer GC have a high affinity for DAPI at $P/D = 1000$.

The amplitude of the autocorrelation function decreases by only a factor of three, while the dye concentration was increased by almost two orders of magnitude. The fluctuation amplitude of each species is weighted by its fractional intensity squared (Eq. 1). If DAPI at a $P/D = 15$ would be bound to the same distribution of binding sites as for $P/D = 1000$, then we would expect a decrease of the fluctuation amplitude by about two orders of magnitude. Thus, in order to explain the reduction of the amplitude by only a factor of three, the additional DAPI must have a much lower fluorescence intensity per molecule than the DAPI bound at $P/D = 1000$. The increased DAPI concentration by going from $P/D = 1000$ to 15 results either in dye bound to polymer binding sites with low molecular brightness or in free dye diffusing in solution.

To reduce the size of the aggregates, we performed measurements after heating the samples to their respective melting temperatures and cooling them subsequently. For the homopolymer, contrary to the measurements before the heat treatment, the two P/D ratios examined behaved in different ways. Although both complexes showed a reduction in the average correlation time, the complex at high P/D ratio reduced the correlation time by 1.3 orders of magnitude (3 ms) and only by 1 order of magnitude for the complex at low P/D ratio (Fig. 4A and B). It can be inferred that the heat treatment did not completely dissolve the aggregates. After heat treatment the correlation times were still distributed, although the averaged size of the aggregates was reduced (Fig. 5B). The dependence of the correlation time on the P/D ratio after heat treatment indicates that DAPI bound to the polymer stabilizes the aggregates.

The $G(0)$ extrapolated value of the homopolymer GC at high P/D ratio decreased after heat treatment from 0.12 to 0.054 (Table 3). This reduction of the fluctuation amplitude by a factor of two indicates that the number of independent fluctuating fluorescent entities increased, which is consistent with a reduction of the size of aggregates. The FCS results clearly indicated that the degree of aggregation of the sample was partially modified by the heat treatment, but that most of the aggregates were too tight to be dissolved at high temperature.

For the complexes between GC polymer in alternating sequence and DAPI, at high P/D ratio the autocorrelation curve is modeled remarkably close by a single diffusing species with a correlation time of 0.01 s (Fig. 5). The heat treatment did not change the autocorrelation function significantly. The correlation time decreased by approximately 30% and the fluctuation amplitude decreased by a factor of two (Table 3). Thus, some of the aggregates present in the sample were removed by the heating procedure. At low P/D ratio, the correlation times are very close to the ones observed at high P/D ratio. However, the $G(0)$ value decreased by roughly one order of magnitude upon increasing the DAPI concentration by a factor of about 70 (Table 3). Thus, in contrast to the homopolymer GC, more bright binding sites are available in the case of the alternating GC polymer.

For comparison, we performed FCS measurements on alternating AT sequences/DAPI complexes (Table 3 and Fig. 6). The autocorrelation curve is slightly broader than compared to that of a single diffusing species. Heat treatment reduced the size and number of aggregates. The $G(0)$ value decreased by a factor of 4 and the correlation time decreased by a factor of 6 (Table 3). The autocorrelation function after heat treatment is still broader than compared to that of a single diffusing species (Fig. 6). The same results were obtained with homopolymeric AT sequences/DAPI complexes.

Discussion

According to previous literature reports, the reduction of the fluorescence emission spectrum intensity when DAPI is complexed with polyd(GC), together with a narrowing of the emission profile, suggest that the dye might be located near the surface of the polynucleotide and exposed to the solvent (Barcellona and Gratton 1990; Sehlstedt et al. 1993). On the contrary, we observed a marked increase of fluorescence intensity, by about a factor of 20, associated with the binding of DAPI with GC in the homopolymeric sequence, clearly showing a different type of interaction between DAPI and these two polymers. These differences are further confirmed by the resolution of the fluorescence decay behavior exhibited by DAPI bound to both polynucleotides. Despite the differences in the GC sequences, at least two decay components are always present within the P/D range examined (but their relative contribution is enormously different). This result suggests that DAPI binding changes the degree of the indole ring solvation, as previously reported (Barcellona and Gratton 1990). The change in lifetime is likely due to subtle differences in proton transfer capability upon formation of the complex, whose extent depends on the varying DAPI binding modalities. The pre-existent structural difference among the GC polymers might be the determinant factor for the occurrence of the different binding situations.

The presence of at least two decay components was the only similarity in the DAPI bound fluorescence behavior between the homo and alternating polymer. The relative fractional fluorescence intensities were

signifcantly different. For the polyd(GC)/DAPI complexes the fraction of the long lifetime component has a constant value of about 45% for almost all P/D ratios examined. For the poly(dG)·poly(dC)/DAPI complexes the long lifetime component dominates (from 90% to 70%). When phase and modulation data were analyzed by a continuous distribution of lifetime values of Lorentzian shape, the parameters of the distribution were indicative of a large heterogeneity of binding situations. The recovered narrow shape of the long lifetime component distribution in the complexes with the GC in homopolymeric sequence ($W_1 \cong 0.01$ ns), at all P/D ratios, with respect to wider distribution recovered by the complexes in alternating sequence ($W_1 = 0.2$ ns ($\bar{x}$)), was a clear indication that the heterogeneity of the system cannot be attributed to different binding affinities, but rather to an intrinsic polymer structure. The distribution analysis of the short lifetime component of the GC/DAPI complexes, either homo or alternating sequence, showed a value of the distribution width of 0.4 ns. This result indicated a large heterogeneity of binding situations.

The fluorescence lifetime data were also analyzed by performing a global simultaneous analysis of multiple fluorescent decay data using discrete exponential components. In the global analysis the number of lifetime components was progressively increased from two to four and their value was linked throughout the analysis. This model assumes that each lifetime component corresponds to independent binding sites (Beechem et al. 1991). The pre-exponential factors, i.e., the number of molecules having a given lifetime, were allowed to vary during the fit procedure. Although the value of the global χ^2 was satisfactory when the components increased to 4, a closer inspection of the recovered parameters showed, for the pre-exponential factor of the longer lifetime component, a kind of bimodal pattern, decreasing at intermediate P/D ratios and rising at low P/D ratios (from 4 to 1). Such decrease followed by an increase in the population was incompatible with the hypothesis of the independence of binding sites. Therefore the global analysis restricted the possible scenarios to either a relatively large number (>4) of binding situations or to non-independent binding sites. Furthermore, the relative population of the long lifetime component apparently saturated at very low DNA-dye coverage. This observation per se is sufficient to conclude that there is a binding modality with a stoichiometry such that only a few binding sites exist in the polymer duplex displaying a long lifetime. This high affinity site cannot be an intercalative or minor/major groove site, because there are many of these sites per duplex. This long lifetime component must arise in situations that are relatively rare, such as bulged nucleotides, which occur when extra nucleotides in one strand of the duplex with large polymer aggregate. These situations occur differently for homopolymeric GC sequences (e.g. the formation of a single helical strand at the 5′ or 3′ edge, respectively, or duplex parallel aggregations) compared to alternating GC sequences (e.g. mismatches or misalignment with formation of protruding nucleotides in an extrahelical structure).

Binding to rare structures

The FCS experiments clearly show that DAPI binds to GC-containing polymers as well as to all polydeoxynucleotides, independently of their base content and sequence. The significant differences between the polymers lie in the strength of the interactions with DAPI (higher in the AT polymers) and in the structural variability (homo versus alternating GC sequence). The average fluorescence enhancement is larger for AT polymers than for GC polymers, even if they both induce a large change in the translational mobility of DAPI as detected by the FCS experiments. The major difference between AT- and GC-containing polymers is that for the AT polymers the bright fluorescence complex arises from DAPI bound to the DNA minor groove. In this case, the binding stoichiometry follows a classical binding curve that correlates with the number of bases present. For the GC-containing polymers, the bright fluorescent species arise from relatively large molecular aggregates that provide unusual binding situations. Those molecular aggregates are different in the homo- and in the alternating copolymer. The homopolymeric GC sequence could form a four-stranded, repeated motif high in guanine, called G-quartet structure, in which all strands are parallel (Morgan 1970; McGavin 1971). For this alternative helical form a Höogsteen base-pairing scheme has been suggested (Sen and Gilbert 1988, 1990). This unusual structure might be one of the structures responsible for sequestering DAPI from surrounding interaction and consequently for the fluorescence enhancement observed with the homopolymer. Since the bright fluorescent component populates at very low polymer, dye coverage, it must correspond to rare conformations with very high affinity for DAPI. The absence of reaction kinetics in the FCS measurements also indicates the presence of high affinity and brightly fluorescent binding sites. The long decay component of the alternating GC copolymer could likely involve an uncommon structural organization, where some protruding nucleotides from single mismatched helical strands can form unusual binding sites.

Binding to common sites

The short lifetime component, which is distributed, could arise from interactions of DAPI with a few bases. These binding components need a large concentration of the dye to appreciably populate, indicating a low affinity to these binding sites. Furthermore, there is an abundance of this kind of binding site. The short decay component was observed for both structural classes of polymer/DAPI complexes, at all the P/D ratios

examined, and can be attributed to a non-classical intercalation process as evidenced by a poor quantum yield increase upon binding. Colson et al. (1995) presented results on competition binding experiments between DAPI and other minor groove binders and the intercalating drugs proflavine and SN16713 with the two structurally related GC polymers, where DAPI displaced about one fifth of the acridine molecules from their intercalation sites and, simultaneously, the remaining molecules changed markedly their chromophore orientation to adapt to the target sequence. A quantitative difference in the observed reduced dichroism of DAPI complexed with the two GC polymers, upon competition with the intercalating drugs, was also reported. The observation that an intercalating drug competes with DAPI for binding to GC sites suggests that its interaction with GC sequences may involve an intercalation process rather than major groove binding, as previously reported (Kim et al. 1993).

A non-classical intercalation mode of interaction or π-π stacking interaction was suggested by several literature reports based on electric linear dichroism studies (Colson et al. 1995, 1996), two-dimensional NMR spectroscopy (Trotta et al. 1995, 1996), and fluorescence titration studies on unfused aromatic systems (Strekowski et al. 1992). Moreover, it is known that the base pairs in the DNA molecule are not flat but propeller twisted (Chou and Johnson 1993; Kang and Johnson 1993) and, since in the GC sequences stacking deformations are frequent, DAPI may find a locally highly propeller twisted structure, where a partial intercalation, with its phenylindole chromophore moiety, can occur. In our model, DAPI can rotate to adopt a suitable geometry for insertion in the constrained GC sites that have the capacity to form an intercalation site to accommodate the extended bulky chromophore. It has been pointed out that, because of its small molecular weight and dimension, DAPI can encounter a partial intercalation site more easily than other binding ligands such as Hoechst 33258 (Colson et al. 1995).

Conclusions

Based on the resolution of fluorescence lifetime components, the fluctuation correlation data and the experiments at different polymer, dye coverage, we postulate the existence of supramolecular structure-mediated binding sites, where the dye is restricted to an assembled structural polymer region or conformation. The experimental results obtained from the FCS studies provide conclusive evidence for the existence of a distribution of molecular aggregates. A model in which the long lifetime components arise from different types of binding to the polymer such as intercalation, minor/major groove binding, and/or electrostatic interactions with the polymer backbone must be excluded. The short lifetime component, on the other hand, could originate from the above-mentioned binding interactions.

Our studies have shown that in the GC/DAPI complexes a relatively large fluorescence enhancement arises from unusual and rare structures. These structures form because of the chemical characteristics of these polymers, which allow many intermolecular interactions. New interest recently focused on the biological significance of unusual structures (as, for example, runs of guanines at telomeres forming the parallel four-stranded structure) and on biomedical implications in the rational design of novel antisense, antiviral, and diagnostic strategies. An important question is whether we can discriminate and characterize these unusual structures using DAPI fluorescence. Those considerations are beyond the scope of the present study and will be addressed in further experiments.

Acknowledgements The FCS experiments were performed at the Laboratory for Fluorescence Dynamics at the University of Illinois, funded by National Institutes of Health (RR03155). M.L.B acknowledges a travel grant from the MURST Grant 1999.

References

Alcala JR, Gratton E, Prendergast FG (1987) Resolvability of fluorescence lifetime distribution using phase fluorometry. Biophys J 51:587–596

Aragon SR, Pecora R (1976) Fluorescence correlation spectroscopy as a probe of molecular dynamics. Chem Phys 64:1791–1803

Barcellona ML, Gratton E (1989) Fluorescence lifetime distribution of DNA-4′,6-diamidino-2-phenylindole complex. Biochim Biophys Acta 993:174–178

Barcellona ML, Gratton E (1990) The fluorescence properties of a DNA probe, 4′,6-diamidino-2-phenylindole (DAPI). Eur Biophys J 17:315–323

Beechem JM, Gratton E, Ameloot M, Knutson JR, Brand L (1991) The global analysis of fluorescence intensity and anisotropy decay data: second-generation theory and programs. In: Lakowicz JR (ed) Topics in fluorescence spectroscopy, vol 2. Plenum Press, New York, pp 241–305

Berland KM, So PT, Gratton E (1995) Two-photon fluorescence correlation spectroscopy: method and application to the intracellular environment. Biophys J 68:694–701

Berland KM, So PT, Chen Y, Mantulin WW, Gratton E (1996) Scanning two-photon fluctuation correlation spectroscopy: particle counting measurements for detection of molecular aggregation. Biophys J 71:410–420

Björling S, Kinjo M, Földes Papp Z, Hagman E, Thyberg P, Rigler R (1998) Fluorescence correlation spectroscopy of enzymatic DNA polymerization. Biochemistry 37:12971–12978

Chen Y, Müller JD, Berland KM, Gratton E (1999) Fluorescence fluctuations spectroscopy. Methods 19:234–252

Chiang SY, Welch J, Rausher FJ, Beerman TA (1994) Effects of minor groove binding drugs on the interaction of TATA box binding protein and TFIIA with DNA. Biochemistry 33:7033–7040

Chou PJ, Johnson WC Jr (1993) Basic inclinations in natural and synthetic DNAs. J Am Chem Soc 115:1205–1214

Coll M, Frederick CA, Wang AHJ, Rich A (1987) A bifurcated hydrogen-bonded conformation in the d(A·T) base pairs of the DNA dodecamer d(CGCAAATTTGCG) and its complex with distamycin. Proc Natl Acad Sci USA 84:8385–8389

Coll M, Aymami J, van der Marel GA, van Boom JH, Rich A, Wang AH (1989) Molecular structure of the

netropsin-d(CGCGATATCGCG) complex DNA conformation in an alternating AT segment. Biochemistry 28:310–320
Colson P, Houssier C, Bailly C (1995) Use of electric linear dichroism and competition experiments with intercalating drugs to investigate the mode of binding of Hoechst 33258, Berenil and DAPI to GC sequences. J Biomol Struct Dyn 13:351–366
Colson P, Bailly C, Houssier C (1996) Electric linear dichroism as a new tool to study sequence preference in drug binding to DNA. Biophys Chem 58:125–140
Dann O, Bergen G, Demant E, Volz G (1971) Trypanosomicidal diamidines of 2-phenylbenzofuran, 2-phenylindene, 2-phenylindole. Justus Liebigs Ann Chem 749:68–89
Eigen M, Rigler R (1994) Sorting single molecules: application to diagnostics evolutionary biotechnology. Proc Natl Acad Sci USA 91:5740–5747
Elson EL, Madge D (1974) Fluorescence correlation spectroscopy. I. Conceptual basis and theory. Biopolymers 13:1–27
Eriksson S, Kim SK, Kubista M, Nordén B (1993) Binding of 4′,6-diamidino-2-phenylindole (DAPI) to AT regions of DNA: evidence for an allosteric conformational change. Biochemistry 32:2987–2998
Grant RC, Harwood SJ, Wells RD (1968) The synthesis and characterization of poly d(I-C)poly·d(I-C). J Am Chem Soc 90:4474–4476
Gratton E, Limkeman M (1983) A continuously variable frequency cross-correlation phase fluorometer with picosecond resolution. Biophys J 44:315–324
Gratton E, Limkeman M, Lakowicz, JR, Maliwal B, Cherek H, Laczko G, Limkeman M (1984) Resolution of mixtures of fluorophores using variable-frequency phase and modulation data. Biophys J 46:479–486
Kang H, Johnson WC (1993) Linear dichroism demonstrates that the bases in polyd(AC)·polyd(GT) and polyd(AG)·polyd(CT) are inclined from perpendicular to the helix axis. Biochem Biopolym 33:245–253
Kim SK, Eriksson S, Kubista M, Nordén B (1993) Interaction of 4′,6-diamidino-2-phenylindole (DAPI) with poly[d(G-C)$_2$] and poly[d(G-m^{5}C)$_2$]: evidence for major groove binding of a DNA probe. J Am Chem Soc 115:3441–3447
Kinjo M, Rigler R (1995) Ultrasensitive hybridization analysis using fluorescence correlation spectroscopy. Nucleic Acids Res 10:1795–1799
Kinjo M, Nishimura G, Kojama T, Mets U, Rigler R (1998) Single-molecule analysis of restriction DNA fragments using fluorescence correlation spectroscopy. Anal Biochem 260:166–172
Klevit RE, Wemmer DE, Reid BR (1986) ^{1}H NMR studies on the interaction between distamycin A and a symmetrical DNA dodecamer. Biochemistry 25:3296–3303
Klinger J, Friedrich T (1997) Site specific interaction of thrombin and inhibitors observed by fluorescence correlation spectroscopy. Biophys J 73:2195–2200
Madge D, Elson EL, Webb WW (1974) Fluorescence correlation spectroscopy. II. An experimental realization. Biopolymers 13:29–61
Maiti S, Haupts U, Webb WW (1997) Fluorescence correlation spectroscopy diagnostics for sparse molecules. Proc Natl Acad Sci USA 94:11753–11757
Manzini G, Barcellona ML, Avitabile M, Quadrifoglio F (1983) Interaction of diamidino-2-phenylindole (DAPI) with natural and synthetic nucleic acids. Nucleic Acids Res 11:8861–8876
Manzini G, Xodo L, Barcellona ML, Quadrifoglio F (1985) Interaction of DAPI with double-stranded ribonucleic acids. Nucleic Acids Res 13:8955–8967
McGavin S (1971) Models of specifically paired like (homologous) nucleic acid structures. J Mol Biol 55:293–298
Meseth U, Wohland T, Rigler R, Vogel H (1999) Resolution of fluorescence correlation measurements. Biophys J 76:1619–1631
Morgan AR (1970) Model for DNA replication by Kornberg's DNA polymerase. Nature 227:1310–1313
Ornstein RD, Rein R, Breen DL, MacElroy RD (1978) An optimized potential function for the calculation of nucleic acid interaction energies. Biopolymers 17:2341–2360
Palù G, Valisena S, Barcellona ML, Masotti L (1987) DAPI-pUC8 complex: a tool to investigate biological effects of nucleic acid-drug interaction. Biochem Biophys Res Commun 145:40–45
Parolin C, Zanotti G, Palù G (1995) A model for sequence-dependent DNA binding of 4′,6-diamidino-2-phenylindole (DAPI). Biochem Biophys Res Commun 208:332–338
Patel DJ, Canuel LL (1978) Anthracycline antitumor antibiotic nucleic acid interactions. Structural aspects of the daunomycin poly(dA-dT) complex in solution. Eur J Biochem 909:247–254
Rigler R, Földes Papp Z, Meyer Almes FJ, Sammet C, Völcker M, Schnetz A (1998) Fluorescence cross-correlation: a new concept for polymerase chain reaction. J Biotechnol 63:97–109
Rippe K (2000) Simultaneous binding of two DNA duplexes to the NtrC-enhancer complex studied by two-color fluorescence cross-correlation spectroscopy. Biochemistry 39:2131–2139
Schwille P, Bieschke J, Oehlenschlager F (1997) Kinetics investigations by fluorescence correlation spectroscopy: the analytical and diagnostic potential of diffusion studies. Biophys Chem 66:211–228
Sehlstedt U, Kim SK, Nordén B (1993) Binding of 4′,6-diamidino-2-phenylindole to [poly(dI-dC)] and [poly(dG-dC)]$_2$: the exocyclic amino group of guanine prevents minor groove binding. J Am Chem Soc 115:12258–12263
Sen D, Gilbert W (1988) Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implication for meiosis. Nature 334:364–366
Sen D, Gilbert W (1990) A sodium-potassium switch in the formation of four-stranded G4-DNA. Nature 344:410–414
Stoll VS, Blanchard JS (1990) Buffers: principles and practice. Methods Enzymol 182:24–38
Straney DC, Crothers DM (1987) Effect of drug-DNA interactions upon transcription initiation at the *lac* promoter. Biochemistry 26:1987–1995
Strekowski L, Mokrosz JL, Wilson WD, Mokrosz MJ, Strekowski A (1992) Stereoelectronic factors in the interactions with DNA of small aromatic molecules substituted with a short cationic chain: importance of the polarity of the aromatic system of the molecule. Biochemistry 31:10802–10808
Szabo AG, Krajcarski DT, Cavatorta P, Masotti L, Barcellona ML (1986) Excited state pK_a behaviour of DAPI. A razionalization of the fluorescence enhancement of DAPI in DAPI-nucleic acid complexes. Photochem Photobiol 44:143–150
Tanious FA, Veal J, Buczak H, Ratmeyer LS, Wilson WD (1992) DAPI (4′,6-diamidino-2-phenylindole) binds differently to DNA and RNA: minor groove binding at AT sites and intercalation at AU sites. Biochemistry 31:3103–3112
Tanious FA, Spychala J, Kumar A, Greene K, Boykin DW, Wilson WD (1994) Different binding mode in AT and GC sequences for unfused-aromatic dications. J Biomol Struct Dyn 11:1063–1083
Thompson RB (1991) Fluorescence-based fiber-optic sensors. In: Lakowicz JR (ed) Topics in fluorescence spectroscopy, vol 2. Plenum Press, New York, pp 45–65
Trotta E, D'Ambrosio E, Ravagnan G, Paci M (1995) Evidence for DAPI intercalation in GC sites of DNA oligomer [d(CGACGTCG)]2: a ^{1}H NMR study. Nucleic Acids Res 23:1333–1340
Trotta E, D'Ambrosio G, Ravagnan G, Paci M (1996) Simultaneous and different binding mechanisms of 4′,6-diamidino-2-phenylindole to DNA hexamer [d(CGATCG)]2. A ^{1}H NMR study. J Biol Chem 271:27608–27614
Weissman M, Schindler H, Feher G (1976) Determination of molecular weights by fluctuation spectroscopy: application to DNA. Proc Natl Acad Sci USA 73:2776–2780
Wells RD, Larson JE, Grant RC, Shortle BE, Cantor CR (1970) Physicochemical studies on polydeoxyribonucleotides containing defined repeating nucleotide sequences. J Mol Biol 54:465–497
Wennmalm S, Edman L, Rigler R (1997) Conformational fluctuations in single DNA molecules. Proc Natl Acad Sci USA 94:10641–10646

Widengren J, Rigler R (1998) Fluorescence correlation spectroscopy as a tool to investigate chemical reaction in solution and on cell surfaces. Cell Mol Biol 44:857–879

Wilson WD, Tanious FA, Barton HJ, Strekowski L, Boykin DW (1989) Binding of 4′,6-diamidino-2-phenylindole (DAPI) to GC and mixed sequences in DNA: intercalation of a classical groove-binding molecule. J Am Chem Soc 111:5008–5010

Wilson WD, Tanious FA, Barton HJ, Jones RL, Fox K, Widra RL, Strekowski L (1990) DNA sequence dependent binding modes of 4′,6-diamidino-2-phenylindole (DAPI). Biochemistry 29:8452–8461

Winkler T, Kettling U, Koltermann A, Eigen M (1999) Confocal fluorescence coincidence analysis: an approach to ultra high-throughput screening. Proc Natl Acad Sci USA 96:1375–1378

Wohland T, Friedrich K, Hovius R, Vogel H (1999) Study of ligand-receptor interaction by fluorescence correlation spectroscopy with different fluorophores: evidence that the homopentameric 5-hydroxytriptamine type 3 As receptor binds only one ligand. Biochemistry 38:8671–8681