**Fluorescence Measurements of Duplex DNA Oligomers under Conditions Conducive for Forming M–DNA (a Metal–DNA Complex)**

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**Introduction**

M–DNA (a metal complex of DNA with millimolar concentrations of Zn$^{2+}$, Co$^{2+}$, or Ni$^{2+}$ and basic pH) has been proposed to undergo electron transfer over long distances along the helix and has generated interest as a potential building block for nanoelectronics. We show that DNA aggregates form under solvent conditions favorable for M–DNA (millimolar zinc and pH = 8.6) by fluorescence correlation spectroscopy. We have performed steady-state Förster resonance energy transfer (FRET) experiments with DNA oligomers conjugated with 6-carboxyfluorescein and tetramethylrhodamine to the opposite ends of double-stranded DNA (dsDNA) molecules. Enhanced acceptor emission is observed for distances larger than expected for identical DNA molecules with no zinc. To avoid intermolecular FRET, the fluorescently labeled dsDNA is diluted with a 100-fold excess of unlabeled dsDNA. The intramolecular FRET efficiency increases 25-fold for a 30-mer doubly labeled duplex DNA molecule upon addition of millimolar concentrations of zinc ions. Without zinc, this oligomer has less than 1% FRET efficiency. This dramatic increase in the FRET efficiency points to either significant changes in the Förster radius or fraying of the ends of the DNA helices. The latter hypothesis is supported by our experiments with a 9-mer that show dissociation of the duplex by zinc ions.

While it is well-known that metallic salts serve to assist in the folding of nucleic acid secondary and tertiary structures by screening the negative charges of the phosphate backbone, certain metal ions can destabilize double-stranded DNA (dsDNA) and prevent hybridization by binding directly to the nucleobases (e.g., Cu$^{2+}$ and Zn$^{2+}$). Zn$^{2+}$ ions bind directly to the nucleobases, and it has been shown that Zn$^{2+}$ also facilitates cross-linking of DNA nucleobases freely diffusing in solution. Apparently Zn$^{2+}$ ions form bonds directly with the nucleobases of DNA, disrupting the hydrogen bonds and stacking interactions.

Lee and co-workers have proposed that M–DNA promotes electron transfer over long distances; however, electron transfer through M–DNA has been disputed in the literature. For instance, it was observed that the electron-transfer rate through DNA is dramatically enhanced upon addition of Zn$^{2+}$ ions for DNA molecules bound to a Au surface, but recently that observation was found to occur for Mg$^{2+}$ ions, which do not form metal–DNA complexes, and the increased electron transfer for M–DNA was attributed to an artifact (divalent cations facilitate penetration of the redox probe tip into the DNA monolayer). Furthermore, two other studies have reported experimental evidence that suggests M–DNA is an insulator.

In the initial electron-transfer experiments, double-stranded DNA oligomers were covalently labeled on opposite ends with an electron donor (fluorescein) and an electron acceptor (rhodamine). The extent of quenching of the donor fluorescence in the presence and absence of the acceptor for increasing lengths of labeled DNA and varying concentrations of zinc was measured. Measurable quenching of the donor was reported in experiments using a different electron acceptor whose absorption spectrum did not overlap with the donor fluorescence, efficient donor quenching was also observed for large donor–acceptor distances and was attributed to electron transfer.
We present evidence that upon M–DNA formation the FRET efficiency of a 30-mer dsDNA labeled with fluorescein and rhodamine on opposite ends increases dramatically. In the absence of conditions for M–DNA formation, the FRET efficiency from fluorescein to rhodamine bound to opposite ends of the 30-mer dsDNA is only a few percent or less. Under conditions of high Zn²⁺ concentrations at basic pH, which is conducive for M–DNA formation, the DNA (and Zn hydroxides) aggregate and precipitate. We could usually notice the aggregation by eye, and fluorescence correlation spectroscopy (FCS) experiments also always showed extensive aggregation. Therefore, it was necessary to dilute the 100% labeled DNA with a large amount of unlabeled DNA, in order to avoid intramolecular FRET. Because of the aggregate formation, we have made extensive measurements and corrections of the dyes’ spectral properties and the high light scattering under conditions of M–DNA formation in order to make accurate FRET measurements. We show that intramolecular FRET is present under conditions of M–DNA formation, even for lengths of doubly labeled DNA oligomers that show essentially no FRET under normal buffer conditions. However, based on the complete disruption of a 9-mer helix under these conditions, we attribute this increased FRET of the 30-mer (and 15-mer) to a change in the distance between the donor and acceptor caused by a disruption of the helical structure (fraying) of the DNA oligomers at the ends.

We define the formation of M–DNA operationally; that is, we use the ethidium assay1 to define the conditions under which M–DNA is formed. Our FRET and spectroscopic measurements lead to some new interpretations of the mode of interaction of Zn²⁺ with DNA at the concentrations required for M–DNA formation. This may be due to the shorter oligomers used in our study. Many experiments regarding M–DNA—including the NMR and CD spectra discussed above—were performed for longer DNA molecules, although initial reports of electron transfer through M–DNA were performed using oligomers as short as 20 base pairs. Our results present some new insights into the structure of DNA under these high Zn²⁺ concentrations, which bear light on some of the observations previously reported.

Experimental Methods

DNA Sequences and Preparation of the Duplex DNA Molecules. Oligonucleotide sizes of 9-, 15-, and 30-mers, were chosen such that the oligomer lengths are well below the Kratky–Porod persistence length of dsDNA, ~50 nm.18,19 Oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA) as complementary single-stranded DNA molecules with reversed-phase high-performance liquid chromatography purification. Capillary electrophoresis showed greater than 90% purity for the oligos purchased from IDT; thus, no further purification was performed. The FRET donor and acceptor molecules 6-carboxyfluorescein (6-FAM) and tetramethylrhodamine (TAMRA), respectively, were chosen since they are fluorescent probes commonly used for study of nucleic acids. The base sequences and fluorophore label positions for the ssDNA are shown in Chart 1. The sequences were chosen to stabilize the ends, to be somewhat random, to correspond with earlier studies using these dyes, and to avoid hairpin structures.

Complementary sequences that do not carry fluorescent labels were hybridized to make unlabeled dsDNA molecules, and the single strands were also used to make singly labeled oligomers. Excess unlabeled dsDNA was added to our samples in great excess (100×) of the fluorescently labeled dsDNA to prevent close proximity of fluorescent dsDNA molecules within DNA aggregates. Unlabeled single strand DNA (ssDNA) molecules were also annealed with complementary strands bound to a single fluorescent dye to make the singly labeled double-stranded samples (i.e., 6-FAM-only labeled dsDNA or TAMRA-only labeled dsDNA).

Hybridization was carried out by mixing the single strands in 100 mM TRIS with 100 mM NaCl pH 8.6 (this buffer was used for all measurements) and heating to 94 °C for half an hour. The solution was slowly cooled, reaching room temperature in 4 h.

M–DNA Formation Monitored Using the Ethidium Fluorescence Assay. The loss of ethidium fluorescence was used to judge the conversion of B-DNA to M–DNA. M–DNA was formed by titration of microliter volumes of a stock of 25 mM zinc chloride stored at pH 3 into the sample. Checks were performed to ensure that the buffer is capable of maintaining pH 8.6 for all sample conditions used since M–DNA formation is expected to release imino protons into solution.

Fluorescence Correlation Spectroscopy. Two-photon FCS was used to measure the 30-mer labeled duplex DNA at various zinc concentrations. The instrumentation is described elsewhere.20 Briefly, a 1.3 NA, 40× oil objective (Zeiss, Jena) and 40 mW laser power from a Ti:sapphire mode-locked laser (Mira 900; Coherent, Palo Alto, CA) were used. The excitation wavelength was 780 nm, where 6-FAM has a large two-photon cross section.21 FCS data were taken in an eight-well glass slide (Nunc Lab-Tek, Rochester, NY). The microwells were incubated at room temperature with concentrated bovine serum albumin (BSA) solution to coat the slides to help prevent association of DNA with the substrate. The slides were then repeatedly flushed with buffer to remove excess, unbound BSA.

Translational Diffusion Coefficients by Autocorrelation Analysis. The translational diffusion coefficients for individual, labeled dsDNA molecules and for dsDNA aggregates, which include multiple molecules of labeled dsDNA, were determined by analyzing the temporal autocorrelation of fluctuations in fluorescence intensity arising from concentration fluctuations in the excitation volume. The autocorrelation function, G(t), for fluctuations in fluorescence intensity is given by

$$G(t) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t)^2 \rangle} \quad (1)$$

where \(\delta F(t) = F(t) - F_{\text{average}}\), \(F(t)\) is the value of the fluorescence intensity at time \(t\) and \(F_{\text{average}}\) is the temporally averaged fluorescence intensity. The numerator is the covariance of the fluctuation in fluorescence \(\delta F(t)\) from its mean value at time \(t\) with the fluctuation at time \(t + \tau\). The amplitude of the autocorrelation function, \(G(0)\), is related to \(\langle N \rangle\), the average number of particles in the excitation volume, as follows:

$$G(0) = \frac{\lambda}{\langle N \rangle} \quad (2)$$
$y$ is the inherent volume contrast factor, which is dependent on the point spread function (PSF); e.g., 0.076 for a Gaussian—Lorentzian PSF. For nanomolar fluorophore concentrations, $\langle N \rangle$ is sufficiently small to produce large fluctuations in intensity. The rate at which $G(\tau)$ goes to zero is dependent on the rate of fluctuations in the fluorescence signal. For three-dimensional translational diffusion $^{21-25}$

$$G(\tau) = G(0) \frac{1}{1 + (\tau/\tau_{\text{diff}})^2}$$

where

$$\tau_{\text{diff}} = w_z^2/(8D_{\text{trans}})$$

$D_{\text{trans}}$ is the translational diffusion coefficient of the fluorescent species and $w_x$ and $w_z$ are the lateral and axial dimensions of the focal volume. $\tau_{\text{diff}}$ is the diffusion time (the characteristic decay time of the autocorrelation curve for two-photon excitation). The focal volume was determined by fitting the autocorrelation curve for freely diffusing fluorescein with a known translational diffusion coefficient.

**Molecular Brightness by Photon Counting Histogram (PCH) Analysis.** To probe for small aggregates that may not be observed easily by the autocorrelation analysis alone, we used a PCH analysis. Using PCH, we can estimate aggregate size by quantifying the number of fluorophores within individual diffusing dsDNA aggregates. The PCH plot is a histogram of the fluctuations in the fluorescence intensity. An analysis of the PCH gives the probability distribution, $p(k)$, to detect $k$ photon counts per sampling time; that is, it analyzes the distribution of the rate of occurrence of photon counts per selected time interval. The photon counting distribution curves reflect the brightness (photon counts per sampling time per molecule) distribution of the underlying sample population. The PCH analysis may be extended to the case of multiple diffusing species (each of distinct molecular brightness) by convolution of the PCH functions for the individual species. $^{22}$

**Measuring Extinction Coefficients.** Absorption was measured with a UV—vis Lambda 14 spectrometer (Perkin-Elmer, Wellesley, MA). Mathematica (Wolfram Research, Inc., Champaign, IL) routines were written to analyze the spectral data. The absorption spectral shape functions for 6-FAM and TAMRA were determined using singly labeled ds- and ssDNA oligomers. These absorption spectra were then used to fit the absorption spectra of the doubly labeled dsDNA samples and the samples with high scattering. For these measurements, DNA-strand concentrations of approximately 1 $\mu$M were used. These spectra were carried out with or without 45 $\mu$M unlabeled 15-mer dsDNA to check for interstrand interactions that affect the spectral shapes. These spectra were then used to analyze the contributions of the donor and acceptor absorption components to the absorption spectra in the presence of scattered light (high zinc concentrations). Light scattering and DNA absorbance were subtracted by fitting the light scattering profile in regions of the spectrum where the fluorescent dyes do not absorb (see Results). Knowing the concentrations of double-strand and single-strand oligomers of DNA, the fluorophore extinction coefficients could be calculated using Beer’s law

$$\text{OD}(\lambda) = \epsilon(\lambda)bc$$

where $\text{OD}(\lambda)$ is the optical density and $\epsilon(\lambda)$ is the extinction coefficient (M$^{-1}$ cm$^{-1}$) at wavelength $\lambda$, $b$ is the path length (cm), and $c$ is the concentration (moles per liter). Absorption spectra were recorded for each zinc concentration used in the M—DNA experiments to determine whether the extinction coefficients remain constant with changing zinc concentration.

**Steady-State Fluorescence Spectroscopy.** A modified photon-counting fluorometer, ISS-PC (ISS, Champaign, IL), was used to acquire the fluorescence data. All fluorescence measurements were temperature controlled. The temperature was 24 °C for measurements with the 15- and 30-mers. The 9-mer measurements were carried out at 10 °C to ensure its double-stranded structure. The magic angle condition (a 54.7° relative orientation between the excitation and emission polarizers) was used to avoid polarization artifacts in the emission spectra.

Corrected fluorescence emission spectra of 6-FAM were collected from 510 to 750 nm for an accurate determination of the spectral shape (the intensity is zero above 700 nm). A 488 nm “Razor Edge” long wave pass filter (Semrock, Rochester, NY) was necessary to block strong excitation light scattering from the FRET spectra that results from selective excitation of 6-FAM molecules. This ensured accurate calculations at high zinc concentration for measurements that rely on the determination of the donor contribution to the FRET spectrum. Light scattering (without the emission filter) could be observed by scanning the emission monochromator of the fluorometer in the region of the excitation wavelength. Directly excited acceptor (TAMRA) fluorescence emission spectra were acquired from 575 to 750 nm. The emission wavelengths started at least 20 nm higher than the excitation wavelength for all samples in order to minimize scattered light in the emission spectra.

Routines in IgorPro (WaveMetrics, Inc., Portland, OR) were used for all calculations using the fluorescence spectra and for calculating the $\langle \text{OD} \rangle$ (eq 12). All spectra were corrected for the wavelength-dependent response of the emission monochromator and detector. Fluuctuations in the lamp intensity were corrected automatically with a standard quantum counter. Except where noted, the doubly labeled samples were composed of 70 nM doubly labeled DNA with 7000 nM excess unlabeled dsDNA (added to prevent intermolecular FRET within aggregates of the dsDNA).

**Fluorescence Quantum Yields.** Fluorescin, F1300 from Molecular Probes (Invitrogen, Carlsbad, CA), in 0.1 N NaOH was used as a reference fluorophore with a known quantum yield of $\Phi_R = 0.95 \pm 0.03$ at 22 °C. With this standard, the fluorescence quantum yield of 6-FAM conjugated to dsDNA before and after conversion to M-DNA can be determined. The following relation was used to calculate the fluorescence quantum yield of the sample, $\Phi$:

$$\Phi = \Phi_R \frac{I}{I_R} \frac{\text{OD}_R}{\text{OD}} n^2$$

where $n$, $I$, and OD are the refractive index, integrated intensity, and optical density both for the sample and for the quantum yield standard (designated by the subscript R). Concentrations were chosen such that the optical densities were less than 0.05 to avoid inner filter effects. We assumed the refractive index of water ($n = 1.33$) for the sample and for the quantum yield standard. Note the refractive index used for these fluorescence measurements is different than that used for the FRET calculations. The refractive index used for the normal fluorescence spectral measurements corrects for solvent effects affecting the amount of light that reaches the collection lens. On the other hand, the rate of energy transfer is dependent on the local refractive index in the immediate volume surrounding the fluorophore. $^{27}$
Forster Radius Calculation and Expected FRET Efficiencies. We measured the FRET efficiency by monitoring the acceptor emission. The efficiency of energy transfer, $E$, is the quantum yield for the energy transfer process; that is, the ratio of the number of quanta of energy transferred to the acceptor molecules to the number of quanta of energy that are absorbed by the donor molecules. The distance dependence of the energy transfer efficiency for FRET is expressed as

$$E = \left[ 1 + \left( \frac{r}{R_0} \right)^6 \right]^{-1} \quad \text{(7)}$$

where $r$ is the distance between the donor and acceptor and $R_0$ is the Forster radius—the distance between the donor and acceptor at which the probabilities of energy transfer and photon emission are equal.

The Forster radius, in angstroms, is calculated from the orientation factor, $\kappa^2$, the donor fluorescence quantum yield, $\Phi_D$, and the overlap integral (in units of nm$^6$/mol), $J(\lambda)$

$$R_0 = \left( \frac{8.79 \times 10^{-28} \kappa^2 \Phi_D J(\lambda)}{n^4} \right)^{1/6} \quad \text{(8)}$$

where $n$ is the refractive index of the medium surrounding the fluorophores. $n = 1.5$ for dsDNA. The overlap integral is calculated from the donor fluorescence emission spectrum and acceptor absorption spectrum

$$J(\lambda) = \frac{10^{17} \epsilon_{A,\text{max}} \int_0^\infty \left( \epsilon_A(n(\lambda) \lambda^2) f_D(\lambda) \right) d\lambda}{\int_0^\infty f_D(\lambda) d\lambda} \quad \text{(9)}$$

where $\epsilon_{A,\text{max}}$ is the maximum extinction coefficient of the acceptor molecule in units of M$^{-1}$ cm$^{-1}$, $\epsilon_A(\lambda)$ is the normalized absorption spectrum of the acceptor, and $f_D(\lambda)$ is the donor fluorescence emission shape spectrum. The peak value of the emission spectrum should be chosen such that the area of $f_D(\lambda)$ is unity. Alternatively, as shown here, the denominator of $\int_0^\infty f_D(\lambda) d\lambda$ normalizes the overlap integral by the area of the fluorescence emission spectrum.

The absorption spectra of TAMRA labeled 15- and 30-mer dsDNA during a zinc titration and the fluorescence emission spectrum from DNA labeled with 6-FAM (corrected for the instrument response) were measured to calculate the overlap integral. The measured fluorescence quantum yields of 6-FAM and measured extinction coefficients of TAMRA for normal DNA and M$^-$DNA were used. A helical model of dsDNA was used to predict the donor-to-acceptor dye separation, $r$. The calculated $R_0$ and $r$ can then be used to calculate the expected FRET efficiency; see eq 7.

(ratio)$_\lambda$ Method for Empirical Determination of FRET Efficiencies. A sensitive measure of FRET is the (ratio)$_\lambda$, a ratio-metric method that employs the fluorescence spectra of the sample. The (ratio)$_\lambda$ is an indicator of the enhanced acceptor emission intensity resulting from FRET and is normalized by the directly excited acceptor emission intensity

$$\text{(ratio)$_\lambda$} = \frac{\sum_{\text{em}} F_{\text{DA}}(\lambda_{\text{em}}^\text{exc} - \lambda_{\text{em}}^{\lambda}) - F_{\text{D}}(\lambda_{\text{em}}^{\lambda})}{\sum_{\text{em}} F_{\lambda}(\lambda_{\text{em}}^{\lambda})} \quad \text{(10)}$$

$F_{\text{DA}}$, $F_{\text{D}}$, and $F_{\lambda}$ are the fluorescence intensity of the FRET pair, the donor only, and the directly excited acceptor fluorescence, respectively, for the emission wavelength $\lambda_{\text{em}}$ and the excitation wavelengths $\lambda_{\text{em}}^{\lambda}$ and $\lambda_{\text{em}}^{\lambda}$, corresponding to direct excitation of the donor or acceptor molecules. Examples of fluorescence emission spectra needed to calculate the (ratio)$_\lambda$ are shown (Figure 1). The notation for eq 10, $\sum_{\text{em}}(...)$, indicates that we sum over a discrete set of data points from an appropriate range of the emission spectra. Note that $F_{\text{DA}}(\lambda_{\text{em}}^\text{exc} - \lambda_{\text{em}}^{\lambda})$ and $F_{\lambda}(\lambda_{\text{em}}^{\lambda})$ are measured from the same sample. $F_{\lambda}$ is an internal normalization factor for each sample that corrects for variations in the acceptor fluorophore concentration and fluorescence quantum yield. This is critical when comparing (ratio)$_\lambda$ values taken from different samples. Hence, the (ratio)$_\lambda$ method is particularly advantageous for M$^-$DNA studies for which the fluorescence quantum yields are strongly affected by the various sample conditions used in the experiments. In addition, there are many mechanisms by which the fluorescence of a donor (fluoroscein) can be quenched; however, the enhancement of the acceptor (rhodamine) is probably the best evidence for FRET.

Figure 1. Example fluorescence spectra used for calculating the (ratio)$_\lambda$. The vertical axes are emission intensities normalized by the lamp and horizontal axes are the emission wavelengths. The fluorescence spectra needed to calculate the (ratio)$_\lambda$ are as follows: (1) the doubly labeled sample emission excited at 485 nm (only every third data point is shown for clarity); (2) the acceptor emission for the doubly labeled sample directly excited at 555 nm; (3) the normalized donor emission for the singly labeled sample excited at 485 nm; (4) the extracted acceptor emission by subtracting (3) from (1). (a) Doubly labeled 30-mer dsDNA molecules with a 100-fold excess of unlabeled dsDNA. (b) Doubly labeled 30-mer dsDNA with a 1000-fold excess of unlabeled dsDNA after addition of 4.5 mM zinc to the sample (to form M$^-$DNA). The ratio of the extracted acceptor emission (4) to the directly excited acceptor emission (2), the (ratio)$_\lambda$, is dramatically increased in the presence of Zn$^{2+}$ ions, indicating increased FRET efficiency. Note that the emission intensity of TAMRA (2) also increases dramatically in the presence of Zn$^{2+}$ ions.
resulting from direct excitation of the acceptor at the donor fluorophore’s peak absorption wavelength is also present in the numerator. However, this is subtracted, as in eq 11, when calculating the FRET efficiency from the (ratio)\(_A\).

The energy transfer efficiency is

\[ E = \frac{\gamma + \beta}{\gamma} \frac{\epsilon_d(555)}{\epsilon_A(485)} \left( \text{(ratio)}_A - \frac{\epsilon_A(485)}{\epsilon_A(555)} \right) \tag{11} \]

where \(\epsilon_d\) and \(\epsilon_A\) are the extinction coefficients of the donor and acceptor, at the excitation wavelength given in parentheses in nanometers. Excitation at 485 nm was used to selectively excite 6-FAM while 555 nm was used for selective excitation of TAMRA. The fraction of DNA molecules doubly labeled with donor and acceptor and DNA molecules singly labeled with acceptor molecules are represented by \(\gamma\) and \(\beta\), respectively. For preparation of the dsDNA samples, the concentration of single strands labeled with donor was 10–20% larger than that of single strands labeled with acceptor. This was done to ensure that a negligible amount of acceptor-labeled single-strand DNA molecules are left over from the hybridization reaction. This procedure biases the factor \((\gamma + \beta)/\gamma\) of eq 11 toward unity, simplifying interpretation of the (ratio)\(_A\). Note that the fraction \((\gamma + \beta)/\gamma\) can be simplified to \((d^+)^{-1}\), where \(d^+\) is the percent donor labeling of the donor-labeled single strands within the sample, as expressed in the original derivation.\(^{31}\)

Results

Ethidium Fluorescence Assay Shows the Formation of M–DNA. An ethidium bromide assay was used initially to test for the binding of zinc to dsDNA. Ethidium was excited at 523 nm, and emission was collected at 604 nm. The ethidium fluorescence assay was used to monitor formation of M–DNA at various concentrations of zinc for unlabeled 15-mer dsDNA molecules. Our ethidium fluorescence kinetics measurements show that conversion to M–DNA is complete within about a half hour at 2 mM zinc, pH 8.6 (Figure 2). Below 2 mM zinc the transition to M–DNA is incomplete (as shown for 1 mM zinc in Figure 2). The ethidium fluorescence is rapidly restored by EDTA, a chelator with high affinity for divalent cations.

The peak fluorescence emission wavelength of ethidium shifts by EDTA, a chelator with high affinity for divalent cations. \(^{33}\) The ethidium fluorescence is rapidly restored within a half hour (Figure 2). Below 2 mM zinc conversion to M–DNA is incomplete (as shown for 1 mM zinc in Figure 2). The ethidium fluorescence is rapidly restored by EDTA, a chelator with high affinity for divalent cations. The peak fluorescence emission wavelength of ethidium shifts to the red by about 10 nm upon formation of M–DNA, which is expected for free ethidium molecules.\(^{33}\) Hence, we attribute the decrease in ethidium fluorescence to dissociation of the molecules from M–DNA rather than to fluorescence quenching by zinc ions, in accord with the decrease in fluorescence intensity upon dissociation of ethidium from dsDNA in the absence of zinc ions. In contrast to the ethidium bromide assay, we measure an immediate jump in the FRET efficiency at 2 mM zinc.

DNA Aggregation Detected by Fluorescence Correlation Spectroscopy. The autocorrelation and PCH analyses of the FCS data revealed that aggregates of dsDNA form in our samples at the zinc concentration required for conversion from B-DNA to M–DNA (Figures 3 and 4). Furthermore, our data suggest that M–DNA molecules associate with the metal hydroxide precipitant; hence, apparently M–DNA formation is concomitant with the coprecipitation of dsDNA molecules and zinc hydroxide. This result is conclusive as evidenced by the following observations: (1) the autocorrelation analysis indicates large diffusing objects at the millimolar zinc concentrations needed to form M–DNA; (2) the excitation light scattering amplitude observed in a fluorometer shows large increases in scattering at the same concentrations of zinc where the diffusion coefficient was observed to decrease dramatically; (3) PCH analysis revealed that many fluorescent molecules are present within each of the diffusing particles, i.e., several dsDNA molecules per precipitant; and (4) at higher zinc concentrations (~5 mM) the precipitant is visible by eye and the supernatant removed from a precipitated sample by centrifugation has negligible fluorescence, suggesting that the dsDNA molecules are part of the precipitant.

Extinction Coefficients Determined from Absorption Spectroscopy. According to eq 11, the ratios of the donor and acceptor extinction coefficients are needed for calculating the FRET efficiencies from the (ratio)\(_A\) values. We determined the extinction coefficients and the extinction coefficient ratios by extracting the absorbance spectra of the fluorophores from the measured absorption traces by subtracting the contributions from light scattering and dsDNA absorption.

For particles with dimensions on the order of the wavelength of incident light, the incident plane wave radiation is mostly scattered (Mie scattering) in the forward direction, because there is more destructive interference in the backward direction.\(^{34}\) Since the average M–DNA aggregate size was estimated from the FCS data to be on the order of the wavelength of light at millimolar zinc concentrations, the wavelength dependence of light scattering in the absorption spectra should follow Mie scattering. The intensity of Mie scattering has a \(\lambda^{-4}\) dependence, where \(x\) is 2, 2.26, or 3, for rods, coils, and spheres, respectively. This is in contrast with Rayleigh scattering which shows a \(\lambda^{-4}\) dependence with particle diameters less than about one-tenth the wavelength of light.\(^{35}\)

To test the wavelength dependence of the scattering from M–DNA samples, we first fit the absorption spectra, OD\(_{scat}(\lambda)\), of our unlabeled M–DNA samples to the function

\[ \text{OD}_{scat}(\lambda) = C \lambda^{-\alpha} \tag{12a} \]

where \(C\) and \(\alpha\) are the scattering amplitude and wavelength dependence, respectively. From the fit we found the wavelength
dependence, $x$, to range from about 2.6 to 2.9 (Figure 5a), depending on the concentration of zinc, indicating the presence of Mie scattering as expected from the average particle size of several hundred nanometers as measured by FCS, which is on the order of the wavelength of light. In practice we found that requiring $x$ to be a fixed value for all samples does not give an optimal fit for every sample, due to small variations among the samples. To correct for scattering, to obtain a better fit of the absorption spectra, we use a polynomial of the following form

$$OD_{scat}(\lambda) = C_1\lambda^{-3} + C_2\lambda^{-2} + C_3\lambda^{-1} + C_4\lambda^{-1}$$

(12b)

$C_i$ and $x$ are the scattering amplitudes and wavelength dependences. In order to extract the extinction coefficient of the dyes for each zinc concentration, the absorption spectra of the fluorescent dyes (in the absence of scattering) were used to fit the absorption spectra together with the above polynomial representation of the scattering function (Figure 5) plus absorption of dsDNA. Thus, the complete function used to fit the absorbance data is

$$OD_{total} = OD_{scat}(\lambda) + A_{dsDNA} P_{dsDNA}(\lambda) + A_{FAM} P_{FAM}(\lambda) + A_{TAMRA} P_{TAMRA}(\lambda)$$

(13)

$A_i$ and $P_i(\lambda)$ are the amplitudes and spectrum profiles for absorbance by dsDNA, 6-FAM, and TAMRA. The spectrum

Figure 3. (a) Autocorrelation curves and (b) photon counting histograms for 15-mer dsDNA singly labeled with 6-FAM are shown for 0, 0.5, and 1 mM zinc ($\square$); 2 mM zinc ($\circ$); and 3 mM zinc ($\bullet$). Fits to the data are included (---). The concentration of labeled DNA molecules was about 15 nM in double strand and the total DNA concentration was 10 μM in base pairs. At 2 mM zinc a large decrease in the diffusion coefficient and increase in the molecular brightness are apparent. Treatment of the diffusing molecules as spheres gives values of the hydrodynamic radii of about 1.6 and 600 nm for the individual 15-mer dsDNA molecules and the zinc hydroxide, 15-mer dsDNA coprecipitant, respectively. The hydrodynamic radii, $R_h$, were calculated using the standard equation for translational diffusion of a spherical molecule:

$$D_{trans} = \frac{k_BT}{6\pi\eta R_h}; D_{trans}$$

is the translational diffusion coefficient in three dimensions, $k_B$ is the Boltzmann constant, $T$ is the temperature, and $\eta$ is the solvent’s viscosity. PCH analysis reveals two separate individual diffusing particles with different brightness values. Compared to the brightness of singly labeled single dsDNA molecules at zero zinc concentration, the brightnesses of the two diffusing components at 2 mM zinc have increased by factors of about 1.3 and 19. The former value is attributed to the increase in the fluorescence quantum yield of 6-FAM for a single 15-mer dsDNA molecule (see Table 1), while the latter value represents the average number of labeled dsDNA molecules in a typical diffusing particle, that is, 14. Since only 2.3% of the duplex DNA molecules are labeled, this means the total number of dsDNA molecules (labeled and unlabeled) per diffusing aggregate would be about 600. Thus, a large number of dsDNA molecules are found within each diffusing aggregate of DNA together with zinc hydroxide, and an average diffusing particle contains multiple labeled molecules, even in the presence of unlabeled DNA molecules. For this reason a large concentration of unlabeled DNA molecules was necessary to avoid intermolecular FRET between labeled molecules.

Figure 4. (a) Translational diffusion coefficients from analysis of the autocorrelation curves of Figure 2a emphasize the dramatic decrease in mobility of the duplex DNA at the zinc concentration required (2 mM) for formation of M−DNA. The expected translational diffusion coefficient for 15-mer dsDNA is about 110 μm$^2$ s$^{-1}$ using the equation for translational diffusion of a rigid rod of length, $L$, and with a hydrodynamic radius, $d$, according to Garcia de la Torre et al.:

$$D_{trans} = \frac{(k_BT)}{(6\pi\eta L)} (\ln(p) + \nu),$$

where $p = L/d$ and $\nu = 0.312 + (0.565/p) + (0.100/p^2)$. The values used for $L$ and $d$ were 65.5 and 26 Å, respectively. Hence, the measured diffusion coefficients approximately agree with the theory at low zinc concentrations. (b) Molecular brightness (photon counts per sampling time per diffusing molecule or aggregate) from analysis of the photon counting histograms of Figure 2b. The molecular brightness of 6-FAM increases (open bars) upon conversion to M−DNA while a second brightness component emerges at millimolar zinc concentrations that we attribute to M−DNA aggregates containing multiple fluorescently labeled DNA molecules (filled bars).
profiles are measured in the absence of zinc and are fixed during the fitting routine (for fitting absorption data at high zinc concentrations), while the amplitudes are adjusted to optimize the fit. Note that in the presence of high concentrations of unlabeled dsDNA, the DNA absorbance becomes significant and must be taken into account when extracting the dye absorption (Figure 5b).

The values of the extinction coefficients were found to be independent of zinc concentration when an excess of unlabeled dsDNA is present. However, in the absence of an excess of unlabeled dsDNA, the shoulder in the low wavelength region of TAMRA’s absorption spectrum becomes significantly strengthened at higher Zn$^{2+}$ concentrations and becomes more prominent at millimolar zinc concentrations (Figure 6). Although the normal low wavelength shoulder is present in the excitation spectrum in the absence of Zn$^{2+}$, this increased shoulder intensity does not take place in the excitation spectrum. Addition of a large excess of unlabeled dsDNA to the samples, which eliminates direct interactions between labeled dsDNA molecules (see (ratio)$_\alpha$ measurements below), eliminated the emergence of the blue shoulder. Therefore, this blue shoulder appears to arise from an interaction between dye molecules when the labeled DNA molecules aggregate. In the presence of a high concentration of unlabeled DNA, the interstrand interaction between dye molecules cannot take place. Thus, the shoulder does not result from interstrand interactions between dye molecules and DNA or from the interaction of the dye molecules with the Zn$^{2+}$ ions and zinc complexes. In fact, this shoulder is present for absorption spectra of concentrated TAMRA molecules in water in which the dye molecules are not fully soluble (data not shown). Therefore, we suspect the blue shoulder is derived from dye–dye interactions in the samples without excess unlabeled DNA. That is, the covalently labeled TAMRA molecules are bound to dsDNA in absence of zinc with their amplitudes determined by the fitting routine.

**Figure 5.** Example of the absorption measurements and fitting routine for determining the extinction coefficients of 6-FAM and TAMRA bound to dsDNA at high zinc concentrations (only every fifth data point is shown for clarity). (a) Absorbance of unlabeled M–DNA molecules at 4.5 mM zinc fitted to Mie scattering in the visible wavelength range. (b) Duplex DNA absorption and fit at a high concentration of DNA. Absorption by the excess, unlabeled dsDNA in our samples is substantial in the visible region of the spectrum and must be taken into account. (c) Data and fit according to eq 13 for doubly labeled 30-mer with an excess of unlabeled dsDNA molecules at 4.5 mM zinc. (d) Extracted 6-FAM and TAMRA composite and individual spectra bound to M–DNA along with the composite trace for scattering and DNA absorption. The extracted spectra are the absorption profiles for the dye molecules bound to dsDNA in absence of zinc with their amplitudes determined by the fitting routine.

**Figure 6.** Normalized absorption spectra of TAMRA bound to 30-mer dsDNA in absence of any excess unlabeled dsDNA in the sample at 0 (–), 2 (- - -), and 4.5 mM (• • •) zinc. The spectra were extracted from the absorption data by subtracting away the scattering component. A blue shoulder emerges during the zinc titration (this shoulder is absent from the fluorescence excitation spectra data not shown thus, the blue shoulder arises from a dark species). The blue shoulder indicates formation of TAMRA excimers at millimolar zinc concentrations. Absorption spectra taken in the presence of an excess unlabeled dsDNA do not show the blue shoulder, thus this complication is avoided for the samples prepared for (ratio)$_\alpha$ measurements.
molecules interact directly due to the high local concentration of the labeled M−DNA aggregates.

The measured values of the extinction coefficient maxima remain constant, within error, for both 6-FAM and TAMRA bound to DNA duplex molecules, throughout the entire range of zinc concentrations. We measured the extinction coefficient ratios $\epsilon_{A(555)}/\epsilon_{D(485)}$ and $\epsilon_{D(485)}/\epsilon_{A(555)}$ to be $1.7 \pm 0.1$ and $0.066 \pm 0.005$, respectively. The extinction coefficient ratios were calculated by averaging together the data points for the fluorescent dyes bound to the 15-mer and 30-mer duplexes since the separate absorption measurements of the 15-mer and 30-mer molecules gave similar values for both ratios.

Controls for the Increased FRET Efficiencies Determined by the (ratio)$_0$ Method. To determine the extent of intermolecular FRET associated with DNA aggregation, singly labeled dsDNA control experiments were performed. The fluorescently labeled M−DNA molecules were singly labeled such that they had the same donor and acceptor dye concentrations as the doubly labeled samples (70 nM dsDNA molecules singly labeled with donor fluorophores and 70 nM dsDNA molecules singly labeled with acceptor fluorophores). This first control demonstrates the substantial amount of intermolecular FRET for fluorescently labeled M−DNA molecules in the absence of excess unlabeled dsDNA (Figure 7).

A second control using singly labeled donor- and acceptor-labeled dsDNA molecules was used to test which ratio of unlabeled to labeled DNA was sufficient to maintain the average distance between labeled M−DNA molecules great enough to eliminate intramolecular FRET. A 100:1:1 (excess unlabeled to 6-FAM labeled to TAMRA labeled) ratio of excess unlabeled dsDNA molecules to singly labeled dsDNA molecules was sufficient. The presence of the excess unlabeled dsDNA molecules reduced the efficiency of intramolecular FRET to a negligible amount (Figure 8) eliminating intramolecular energy transfer. This ratio of unlabeled to labeled dsDNA was used for all the intramolecular FRET experiments.

FRET Measurements of the Doubly Labeled 15-mer and 30-mer Oligomers under Conditions of M−DNA Formation. The control experiment (Figure 8) shows that we can reliably measure the intramolecular FRET efficiencies for the M−DNA samples. We prepared our doubly labeled dsDNA samples with a 100-fold excess of unlabeled dsDNA molecules before titrating zinc into the sample to form M−DNA. Upon formation of M−DNA, at 2 mM zinc, we observe large increases in FRET for both 15-mer and 30-mer molecules (Figure 9). The FRET values calculated from (ratio)$_0$ are compared with expected FRET efficiencies, estimated from known donor−acceptor separations for helical dsDNA oligomers and empirically determined $R_0$ values. The estimated donor−acceptor separations were computed using parameters from a geometrical model of dsDNA, reported previously. The donor−acceptor distances expected for 15-mer duplex and 30-mer dsDNA are 65.5 and 119 Å, respectively. Before the addition of zinc, the expected FRET values agree well with the measured values. At millimolar concentrations of zinc, the measured FRET efficiencies are several-fold higher than the expected values (Table 1 and Table 2).

Strand Dissociation of the 9-mer Helix. A 9-mer duplex was investigated in addition to the 15- and 30-mer duplexes. The 9-mer duplex DNA is partially melted at room temperature under normal buffer conditions; therefore, we performed data collection at 10 °C to ensure a double-stranded structure. The FRET efficiency for the doubly labeled 9-mers with no added zinc was in agreement with the prediction of the helical model.
FRET efficiencies determined by the (ratio)A method (Figure 9). The 15-mer duplex DNA were separated, as evident from the abolition of unlabeled DNA (open bars), excess unlabeled 30-mer ssDNA (gray filled bars), or without any excess 7000 nM excess unlabeled 15-mer dsDNA (black filled bars), with 7000 nM samples each containing 70 nM doubly labeled 9-mer dsDNA with 7000 nM of dsDNA. Near the critical concentration of zinc required for DNA formation, excess DNA does not cause the separation of the 9-mer strands, if the 9-mer strands separated to form the third strand of a triple strand complex. The melting of the 9-mer is observed for all cases (Figure 10), confirming that excess DNA does not cause the separation of the 9-mer strands. The FRET efficiency for the sample without any excess unlabeled DNA also clearly indicates strand separation but does not reach the same low FRET efficiency baseline as the other samples (Figure 10). Aggregation of the single-stranded labeled complementary DNA sequences in the absence of excess unlabeled DNA molecules would allow intermolecular FRET.

**TABLE 1: Expected E from the R₀ and Measured, E from (ratio)A, Intramolecular FRET Efficiencies for the 15-mer Duplex DNA (70 nM Doubly Labeled 15-mer dsDNA with 7000 nM Blank 30-mer dsDNA)**

<table>
<thead>
<tr>
<th>[Zn²⁺]/mM</th>
<th>ε₃₅₅nm/M⁻¹ cm⁻¹</th>
<th>Φₐ</th>
<th>J(λ)/10⁻¹² 10⁶ mol⁻¹</th>
<th>R₀ /Å</th>
<th>E from R₀</th>
<th>(ratio)A</th>
<th>E from (ratio)A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>76000 ± 8000</td>
<td>0.26 ± 0.06</td>
<td>2.7 ± 0.3</td>
<td>44.9</td>
<td>0.09</td>
<td>0.127 ± 0.001</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>76000 ± 8000</td>
<td>0.45 ± 0.07</td>
<td>2.7 ± 0.3</td>
<td>49.0</td>
<td>0.15</td>
<td>0.219 ± 0.008</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>4.5</td>
<td>76000 ± 8000</td>
<td>0.42 ± 0.07</td>
<td>2.7 ± 0.3</td>
<td>48.5</td>
<td>0.14</td>
<td>0.18 ± 0.01</td>
<td>0.19 ± 0.04</td>
</tr>
</tbody>
</table>

The expected FRET efficiency was calculated from the measured R₀ assuming the donor to acceptor fluorophore distance from a helical model of duplex DNA, and indicates the amount of FRET to expect at each zinc concentration if the dsDNA structure remains helical.

**TABLE 2: Expected E from the R₀ and Measured E from (ratio)A, Intramolecular FRET Efficiencies for the 30-mer Duplex DNA (70 nM Doubly Labeled 30-mer dsDNA with 7000 nM Blank 30-mer dsDNA)**

<table>
<thead>
<tr>
<th>[Zn²⁺]/mM</th>
<th>ε₃₅₅ nm/M⁻¹ cm⁻¹</th>
<th>Φₐ</th>
<th>J(λ)/10⁻¹² 10⁶ mol⁻¹</th>
<th>R₀ /Å</th>
<th>E from R₀</th>
<th>(ratio)A</th>
<th>E from (ratio)A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>98000 ± 4000</td>
<td>0.92 ± 0.09</td>
<td>3.5 ± 0.2</td>
<td>57.7</td>
<td>0.01</td>
<td>0.08 ± 0.01</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>98000 ± 4000</td>
<td>0.68 ± 0.02</td>
<td>3.5 ± 0.2</td>
<td>54.8</td>
<td>0.01</td>
<td>0.103 ± 0.007</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>4.5</td>
<td>98000 ± 4000</td>
<td>0.4 ± 0.2</td>
<td>3.5 ± 0.2</td>
<td>50.9</td>
<td>0.01</td>
<td>0.22 ± 0.01</td>
<td>0.25 ± 0.03</td>
</tr>
</tbody>
</table>

The expected FRET efficiency was calculated from the measured R₀ assuming the donor to acceptor fluorophore distance from a helical model of duplex DNA, and indicates the amount of FRET to expect at each zinc concentration if the dsDNA structure remains helical.

**Figure 10.** FRET efficiencies for the 9-mer indicate that the duplexes dissociate during a zinc chloride titration performed at 10 °C. The samples each contain 70 nM doubly labeled 9-mer dsDNA with 7000 nM excess unlabeled 15-mer dsDNA (black filled bars), with 7000 nM excess unlabeled 30-mer ssDNA (gray filled bars), or without any excess unlabeled DNA (open bars).

of dsDNA. Near the critical concentration of zinc required for M-DNA formation, ~2 mM, the two single strands of the 9-mer duplex were separated, as evident from the abolition of FRET efficiencies determined by the (ratio)A method (Figure 10). This was initially unexpected.

9-mer dsDNA, doubly labeled samples were prepared with excess unlabeled double-stranded DNA (15-mer), single-stranded DNA (30-mer), and without any excess unlabeled DNA, to test whether excess DNA in single strand or double strand forms catalyze the separation of the 9-mer. For instance triple helices can form in the presence of divalent ions, such as zinc, for some special sequences of DNA, and this could cause the separation of the 9-mer strands, if the 9-mer strands separated to form the third strand of a triple strand complex. The melting of the 9-mer is observed for all cases (Figure 10), confirming that excess DNA does not cause the separation of the 9-mer strands. The FRET efficiency for the sample without any excess unlabeled DNA also clearly indicates strand separation but does not reach the same low FRET efficiency baseline as the other samples (Figure 10). Aggregation of the single-stranded labeled complementary DNA sequences in the absence of excess unlabeled DNA molecules would allow intermolecular FRET.

**Discussion**

**Aggregation of the M-DNA Samples.** Our dsDNA oligomers aggregate concomitantly with M-DNA formation and the M-DNA forms a coprecipitant with the metal hydroxide. The extent of precipitation in our samples is not surprising since it is well-known that zinc hydroxide precipitates at millimolar zinc concentrations at basic pH. For example, precipitation of zinc hydroxide is used as a precursor for making zinc oxide nanoparticles. However, aggregation of M-DNA molecules has not been emphasized in the literature.

We found that adding a 100-fold excess of unlabeled dsDNA molecules (referring to concentrations of dsDNA molecules) to our samples eliminates intermolecular FRET and allows intramolecular FRET for the doubly labeled dsDNA molecules to be determined. We performed several checks to correct for artifacts caused by the high degree of light scattering in our samples. Light scattering acts to increase the path length for the excitation and emission photons. Because we are using dilute samples (nanomolar dye concentration) the fluorescence emission is not strongly attenuated by the light scattering, as would be the case for the inner filter effect (i.e., highly absorbing media). In other words, the multiply scattered light emerges without noticeable attenuation. We find that the spectral shapes of the fluorescent signals for our samples are the same with and without zinc; thus, light scattering is not a problem when analyzing the spectra.

The (ratio)A cancels scattering effects of the acceptor emission. We measured the OD for our samples to correct for the attenuation of the excitation light in the calculation of (ratio)A. For a path length of 0.2 cm (midway through the 0.4 x 0.4 cm cuvette used for FRET experiments) the attenuation of 485 and 555 nm excitation light is nearly identical.

Our energy transfer measurements by the (ratio)A method unambiguously show energy transfer, and with care to eliminate intermolecular energy transfer, the efficiency of intramolecular FRET could be resolved for the 9-, 15-, and 30-mer M-DNA molecules.

**Sensitivity of FRET to Environmental and Molecular Parameters.** The rate of energy transfer is sensitive to several factors other than the distance between the donor and acceptor fluorescent probes, including the following: the local refractive index of the surroundings within about a wavelength of the fluorescent dyes; the orientation factor, k²; the donor fluorophore’s fluorescence quantum yield; and the spectral overlap integral. Can these factors account for the increased FRET?

We consider these factors in this section, and we conclude that they cannot account for the observed increase in FRET efficiency.

**Refractive Index.** The local refractive index influences the sensitivity of FRET to Environmental and Molecular Parameters. The rate of energy transfer is sensitive to several factors other than the distance between the donor and acceptor fluorescent probes, including the following: the local refractive index of the surroundings within about a wavelength of the fluorescent dyes; the orientation factor, k²; the donor fluorophore’s fluorescence quantum yield; and the spectral overlap integral. Can these factors account for the increased FRET?

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**Discussion**

**Aggregation of the M-DNA Samples.** Our dsDNA oligomers aggregate concomitantly with M-DNA formation and the M-DNA forms a coprecipitant with the metal hydroxide. The extent of precipitation in our samples is not surprising since it
4.5 mM zinc (Table 2) and the donor—acceptor distance is expected to be 119 Å for a helical 30-mer. From eq 7, the $R_0$ that would account for this level of FRET efficiency is $R_0 = r(1/E - 1)^{1/6} = 100$ Å. Thus, the $R_0$ would have to increase from 50.9 to 100 Å to account for the increased FRET if the DNA oligomer retained its helical structure. If we assume a refractive index of 1.5 for the FRET calculations of 1.5, which is the refractive index of DNA, from eq 8 the refractive index that would account for the observed increased FRET would be $n' = nR_0/R_0^{3/2} = 1.5 (100 Å/50.9 Å)^{3/2} = 0.5$. Clearly, this refractive index is impossible. Hence, the refractive index is not the major mechanism responsible for increased FRET.

Changes in the Donor Fluorescence Quantum Yield and Overlap Integral. In Table 1 and Table 2 the expected FRET efficiency values take into account changes in the Förster radius resulting from variations in the donor fluorophore’s fluorescence quantum yield and changes in the overlap integral for the various zinc concentrations. The tables show that consideration of the overlap integral and changes in the fluorescence quantum yields cannot account for the increased FRET at millimolar zinc, especially for the 30-mer where the Förster radius is calculated to decrease for M–DNA. Variation in the overlap integral is negligible even though the fluorescence quantum yield of 6-FAM appears to be very sensitive to its position on the DNA and to the binding of zinc ions to the DNA.

The following is a discussion of the known dye–DNA characteristics and the data, which we used to derive the expected efficiency values in Tables 1 and 2. We conclude that variations of $R_0$ are not responsible for the increase in FRET efficiency under conditions conducive for M–DNA formation.

Both 6-FAM and TAMRA are quenched by DNA bases, while some other dyes (such as Cy3 and Cy5) are not quenched significantly.39 Guanine (dG) is the best electron donor of all the DNA bases and may be efficiently oxidized by a nearby fluorophore in its excited state via photoinduced electron transfer.40 For example, it has been reported that 6-FAM bound near a terminal cytosine (dC) at the 3′-end of an oligomer upon hybridization gives 87% quenching of the fluorescence when the terminal dC–dG base pair is in proximity of the fluorescent dye molecule.41 The magnitude of the fluorescence quenching is sensitive to both the primary and the secondary structure of the DNA near the conjugation site of the fluorophore; for example, 6-FAM bound to dC at the 5′-end of an oligomer results in only 40% quenching upon hybridization.41

For our samples, 6-FAM is bound either to the 3′-end, d(CCC) sequence for 15-mer dsDNA, or to the 5′-end, d(CGG) sequence for 30-mer dsDNA, and TAMRA is bound either to the 3′-end, d(CCA) sequence for 15-mer dsDNA, or to the 5′-end, d(CCC) sequence for 30-mer dsDNA. Upon hybridization the fluorescence from both of the dyes, measured using singly labeled samples, is quenched, presumably by nearby dC–dG base pairs—except for 6-FAM conjugated to d(CGG) for the 30-mer. However, M–DNA formation dramatically reduces quenching for both 6-FAM and TAMRA for the 15-mer as well as for TAMRA for the 30-mer (for example, see Figure 1). We carried out a preliminary study of oligonucleotides labeled with Cy3 and Cy5 and found that these dyes do not show changes in their fluorescence quantum yields (data not shown). This is consistent with the hypothesis that zinc ions interfere with quenching of 6-FAM and TAMRA fluorescence by the terminal dC–dG base pairs. For the case of 6-FAM bound to the 30-mer 5′-end, d(CGG) sequence the nearby guanines apparently quench 6-FAM in both the single-strand and double-strand forms.

TAMRA bound to dsDNA is known to have at least three different states when conjugated to dsDNA with different fluorescence quantum yields and lifetimes plus a substantial dark state population sensitive to temperature and ionic strength.42 Changes in the ionic strength and melting of secondary structure presumably affect the availability of the various binding interactions between TAMRA and the DNA base pairs. Therefore, the metal ion binding may either promote a high fluorescence quantum yield state or, perhaps, block the dark state.42 For instance, the positively charged TAMRA molecules may be repelled from the metal complex in the same fashion as the intercalated ethidium molecules that are expelled from dsDNA upon binding of zinc ions. The increasing quantum yield of TAMRA upon M–DNA formation could prove useful as an assay for monitoring M–DNA formation, in similar fashion to the ethidium fluorescence assay.

Changes in Distance between the Donor and Acceptor Fluorescent Probes Due to DNA Fraying. A reasonable candidate for the increased FRET is a change in donor–acceptor distance. What could lead to this decreasing distance between the donor and acceptor?

DNA Curvature Is Not Expected To Lead to the Increased FRET. DNA curvature has been observed to be induced by divalent cations, e.g., Mg$^{2+}$ and Zn$^{2+}$.43 and this has been predicted to result from nonspecific binding of multivalent cations to the dsDNA major groove.44 However, DNA bending, kinking, and curvature usually require specific sequences, i.e., sequence repeats synchronous with the helical turns. For instance, intrinsically curved dsDNA molecules normally contain A-tracts,45 or other special repeat sequences.46 DNA curvature and kinks have been observed for GGGCCC repeat sequences in phase with the helical repeat. The sequences of our 9-, 15-, and 30-mers, do not contain base pair repeats (or obvious sequences) that are known to be prone to sequence-directed curvature or cation-induced curvature. In addition, the oligomers are not long enough to exhibit significant changes in FRET from local deviations from a straight helical axis. The oligomer dsDNA molecules used for this study are well below the normal persistence length of dsDNA; such short oligomers can generally be approximated as straight rigid rods,47 with minimum deviations, which cannot account for the large increase in FRET efficiency.
The 9-mer Melts Before It Can Form M-DNA, Eliminating FRET. A critically important observation of our FRET experiments was that the 9-mer dsDNA oligomers dissociate into single strands at zinc concentrations near (but slightly below) the critical concentration (~2 mM) of zinc needed to form M-DNA. Visible precipitation or aggregation (as observed by FCS) is not observed for this concentration of zinc for the 9-mer, or any of the polymers. The double-strand structure of the 9-mer DNA is not sufficiently stable at concentrations of zinc needed to form MDNA.

At the higher concentrations of the unlabeled DNA, which we used to hinder intermolecular FRET, it is conceivable that the duplex of the 9-mer could dissociate and that the single strands could interact with the much higher concentration of unlabeled DNA, eliminating the intramolecular FRET. However, the dissociation of the 9-mer, leading to the elimination of FRET, does not depend on the presence of the unlabeled DNA (Figure 10). Therefore, we do not consider such intermolecular interactions, to be the cause of the dissociation of the 9-mer oligomers. Also, association of DNA strands that are 100% labeled would not lead to the abolishment of FRET.

Apparently the presence of zinc at millimolar concentrations lowers the stability of the 9-mer duplex DNA. This leads to the conclusion that Zn\(^{2+}\) ions interact more favorably with single strands at these concentrations than with the duplex DNA. This favorable interaction with single strands is apparently large enough so that the 9-mer dissociates completely (but not the 15-mer and 30-mer). Indeed it has been found that Zn\(^{2+}\) ions as well as Cu\(^{2+}\) ions interact with the bases (albeit with different affinities and modes of interaction), and this can lead to decreasing stability of duplex DNA.\(^{10}\) However, the molecular interactions responsible for the variable stabilities are complex; Zn\(^{2+}\) binding can lead to more extensive renaturation of melted DNA samples, lowering the renaturation \(T_m\), and there is evidence that kinetic processes are influenced by Zn\(^{2+}\) interactions.\(^{10}\)

Fraying Could Lead to Increased FRET for 15-mer and 30-mer. We consider the increased FRET for the 15-mer and the 30-mer in light of the dissociation of the 9-mer. As with all short helical oligomers according to statistical zipper models of the helix–coil transition,\(^{48}\) we expect any dissociation of the strands would start by “unzipping” of the helix from the ends of the dsDNA molecules, leading to fraayed helices. Due to the short helical length, even minor fraying of the 9-mer helix would greatly destabilize strand association and could lead to the complete dissociation we observe with the 9-mer. For the 15-mer and 30-mer, the extent of fraying is apparently not sufficient to lead to complete dissociation. But fraying at the ends could lead to a decrease in the distance between the donor and acceptor dye molecules. This would account for the observed increase in FRET for the 15-mer and 30-mer. Interestingly, the destabilizing Zn-DNA interactions discussed in the last subsection are particularly prevalent for poly[(dGdC)]\(_2\);\(^{10}\) our sequences all have GC base pairs at the ends of the helices, which were originally inserted to stabilize the oligomers at the ends to avoid fraying under normal ion conditions.

The properties of M-DNA have been studied previously using atomic force microscopy (AFM).\(^{16}\) It was found that the diameter of dsDNA molecules (10 667 base pairs in length) increases to 6 nm in diameter, and becomes typically 5-fold shorter in length after conversion to M-DNA.\(^{16}\) The AFM images clearly show a shortened and thickened appearance of M-DNA. According to our results, high concentrations of zinc would destabilize the local helix structure, and this could lead to the observed compaction. Moreno-Herrero et al. also reported that M-DNA retains a net charge, based on the ability to capture M-DNA on a mica substrate; however, their M-DNA would not enter an agarose gel matrix with ~50 nm pores. They conclude that the M-DNA cannot enter the agarose gel matrix due to steric inhibition. This is consistent with our observation of aggregation of M-DNA molecules in solution. Also, our results indicating partial dissociation of the double-stranded structure could explain the shortened DNA structures observed in AFM experiments.

Other Studies of Metal-Induced DNA Aggregation. Correlated fluctuations in loosely associated multivalent ions near the polyanionic surfaces of DNA molecules can cause inter- and intramolecular attractions.\(^{49}\) DNA condensation (the orderly compaction of DNA into distinct morphologies such as toroidal DNA) by spermidine\(^{3+}\), spermine\(^{4+}\), and Mn\(^{2+}\) have all been observed.\(^{49,50}\) Supercoiled DNA is condensed into toroids with Mn\(^{2+}\) (which like Zn\(^{2+}\) binds to DNA bases at higher concentrations), whereas linear DNA molecules form disordered aggregates.\(^{50}\) These studies are usually carried out at pH values less than 7.

Raman spectroscopic studies of DNA structure in the presence of transition metal cations (including Cu\(^{2+}\) and Ni\(^{2+}\)) showed that the metal cations initially bind via the major groove to the nucleobases and partially melt the duplex DNA molecule\(^{51,52}\) (Zn\(^{2+}\) was not studied). As the duplex melts, metal binding sites on the bases become exposed, and some bases swing out of the helix. Aggregation is concomitant with the melting (especially for high molecular weight DNA and at temperatures near the \(T_m\), and it is proposed that the metal ions mediate interstrand cross-links that eventually lead to a network of cross-linked DNA molecules.\(^{51,52}\) The mechanism of aggregation of DNA molecules in our samples may be related to this mechanism. However, in our case, the aggregation is concomitant with a precipitant formation of Zn(OH)\(_2\). Also, in these other studies, the cross-linking experiments were carried out at low pH (pH = 6.5) and high metal concentration (100 mM) with high molecular weight DNA (>23 kbp) at high DNA concentration (83 mM in bp). In contrast, our experiments were done at basic pH (pH = 8.6), low metal ion concentration (a few millimolar), and with oligomer dsDNA at lower concentration (~200 \(\mu\)M in bp).

Conclusions

We have shown that conversion to M-DNA is concomitant with aggregation of the dsDNA molecules. This caused intermolecular FRET between separate duplex molecules. Addition of excess unlabeled dsDNA molecules to the samples eliminated intramolecular FRET, which was always present in the absence of an excess of unlabeled dsDNA molecules, due to this aggregation. After addition of the excess unlabeled DNA and spectroscopic corrections for scattering and spectral shifts, our reported FRET efficiencies are representative of intramolecular FRET.

Our FRET measurements show dramatically increased intramolecular FRET for the doubly labeled 15-mer and 30-mer molecules upon formation of M-DNA at millimolar Zn\(^{2+}\) concentrations. In contrast, the FRET efficiency for a doubly labeled 9-mer duplex (which initially had the most FRET efficiency) decreases to zero at zinc concentrations lower than that required for conversion to M-DNA. These results demonstrate that Zn\(^{2+}\) completely dissociates the 9-mer duplex; hence, the presence of Zn\(^{2+}\) affects the duplex–single-strand transition for DNA. The short, rigid oligomers used for this study
do not contain sequences known to kink or curve. Consideration of changes in the Förster radius (and consideration of changes in absorption coefficients, spectral shifts, and artifacts from scattered light) upon conversion to M–DNA for each of the fluorescently labeled oligomers does not account for the increased FRET. We conclude that fraying of the ends of the helix is the most likely mechanism for the increase in FRET efficiency. According to this interpretation, fraying at the ends of the 15-mer and 30-mer M–DNA molecules decreases the donor–acceptor distance.

Quenching of fluorescein and enhancement of rhodamine will take place with shorter doubly labeled (fluorescein/rhodamine) donor–acceptor distance.

At 1 mM the conversion was 80% complete in 1 mM Zn, even for longer times, whereas it has been earlier (judging from the ethidium assay, only 50% conversion) at a few hundred micromolar concentrations of Zn2+.

Our results are relevant to interpretations of other M–DNA studies. Some of these previous studies have been carried out on very long DNA polymers, usually native DNA structures (but shorter structures have also been used previously); our studies have been carried out on short oligonucleotides. We have found some differences to previous reports. For instance, we have always found precipitation (see Figure 3 and Figure 4 and was often visible by eye) under the conditions for M–DNA formation (that is, the ethidium assay showed dissociation of ethidium), even for the low DNA concentrations used in this study. This is in contradiction to earlier reports, which also probed short DNA oligomers (20-mers and longer). There it is reported that precipitation takes place at 1–2 mM base pairs at 1–2 mM Zn2+, but at lower concentrations of DNA, precipitation is reported not to be of concern. We showed that aggregation does take place by FCS. We were also unable to form M–DNA (again judging from the ethidium fluorescence assay) at a few hundred micromolar concentrations of Zn2+, as had been reported previously. However, in contrast to this paper, an earlier report by Lee et al. also showed incomplete conversion. We do not see complete conversion to M–DNA (judging from the ethidium assay, only 50% conversion) at 1 mM Zn, even for longer times, whereas it has been earlier reported that at 1 mM the conversion was 80% complete in 2 h (50% conversion at 0.5 mM). The conversion from M–DNA to normal B-DNA when the Zn2+ was complexed with a chelator (recovery of ethidium fluorescence) was always fairly rapid; we were not able to retain the M–DNA structure when the concentration of Zn2+ was lowered (by addition of chelator).

The strand dissociation of the 9-mer at higher Zn2+ concentrations also emphasizes the instability of the DNA helix under these conditions. This could be a major factor also in experiments with longer DNA strands, where one is interpreting the conductivity and electron-transfer characteristics of the M–DNA. That is, it is possible that certain sequences in the longer strands are not fully helical—this together with the aggregation that we always observed is germane to interpretations of the electrical characteristics of M–DNA. In addition, there must be large amounts of Zn salts surrounding the DNA, which could contribute to increased conductivity. With the FRET assay we can determine if our duplexes are at least partially intact, and the scattering and FCS measurements show unequivocally the aggregation. If the shorter lengths of DNA that we have used aggregate and become partially unstable, it seems reasonable that longer sequences will also aggregate under these conditions and perhaps undergo partial local melting.

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