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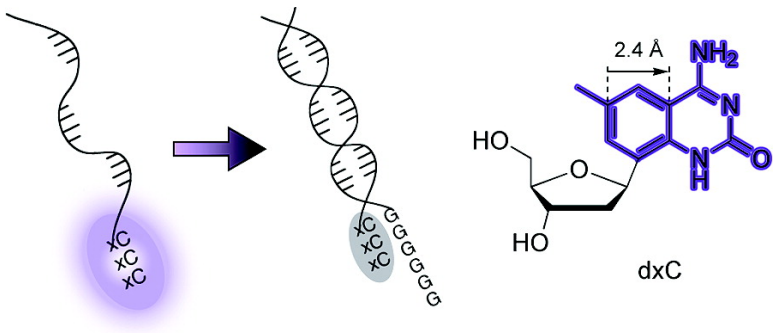
J. Am. Chem. Soc., **2008**, 130 (12), 3989-3999 • DOI: 10.1021/ja0782347



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Fluorescence of Size-Expanded DNA Bases: Reporting on DNA Sequence and Structure with an Unnatural Genetic Set

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Abstract: We recently described the synthesis and helix assembly properties of expanded DNA (xDNA), which contains base pairs 2.4 Å larger than natural DNA pairs. This designed genetic set is under study with the goals of mimicking the functions of the natural DNA-based genetic system and of developing useful research tools. Here, we study the fluorescence properties of the four expanded bases of xDNA (xA, xC, xG, xT) and evaluate how their emission varies with changes in oligomer length, composition, and hybridization. Experiments were carried out with short oligomers of xDNA nucleosides conjugated to a DNA oligonucleotide, and we investigated the effects of hybridizing these fluorescent oligomers to short complementary DNAs with varied bases opposite the xDNA bases. As monomer nucleosides, the xDNA bases absorb light in two bands: one at ~260 nm (similar to DNA) and one at longer wavelength (~330 nm). All are efficient violet-blue fluorophores with emission maxima at ~380−410 nm and quantum yields (Φ_{fl}) of 0.30−0.52. Short homo-oligomers of the xDNA bases (length 1−4 monomers) showed moderate self-quenching except xC, which showed enhancement of Φ_{fl} with increasing length. Interestingly, multimers of xA emitted at longer wavelengths (520 nm) as an apparent excimer. Hybridization of an oligonucleotide to the DNA adjacent to the xDNA bases (with the xDNA portion overhanging) resulted in no change in fluorescence. However, addition of one, two, or more DNA bases in these duplexes opposite the xDNA portion resulted in a number of significant fluorescence responses, including wavelength shifts, enhancements, or quenching. The strongest responses were the enhancement of $(xG)_n$ emission by hybridization of one or more adenines opposite them, and the quenching of $(xT)_n$ and $(xC)_n$ emission by guanines opposite. The data suggest multiple ways in which the xDNA bases, both alone and in oligomers, may be useful as tools in biophysical analysis and biotechnological applications.

Introduction

The design and synthesis of novel DNA base analogues has been the target of considerable research over the past decade. Many base analogues have been designed with intention of expanding the genetic alphabet,[1] studying DNA assembly and replication,[2] or finding new tools for research,[3] all aimed at altering or enhancing function in the context of the natural genetic system. Our laboratory has recently explored an alternative approach to modifying DNA, by replacing all the natural bases with a new set of expanded base analogues, each of which contains an added benzene ring.[4,5] These size-expanded (xDNA) bases retain the Watson−Crick hydrogen-bonding motif and are capable of selectively recognizing their natural Watson−Crick partners.[6] This recognition gives rise to expanded base pairs, extended by 2.4 Å relative to a natural pair due to the benzo-fusion. Single xDNA pairs in the context of natural DNA have been investigated,[7] and fully expanded xDNA helices, which are more stable than natural DNA, have been documented as well.[8] The biophysical and biochemical functions of this DNA mimetic are being explored as an alternative genetic set.

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In addition to the unusual stability and structure of xDNA, the extended conjugation imparted by the benzene ring insertion renders the xDNA bases fluorescent in aqueous buffers,[7,9] a property virtually absent in natural DNA. Because of the ability of xDNA bases to interact with the natural genetic system via their Watson−Crick hydrogen-bonding edges, their inherent fluorescence makes these bases potentially useful both as basic probes of biological mechanisms and interactions and as biotechnological reporters, such as for specific DNA sequence. Although many fluorescent labels have been tethered to DNA, fewer fluorophores are available as nucleobase replacements for probing structure, dynamics, and mechanisms directly within the helical stack.[3c−e,10] In addition, there are only rare examples wherein the use of multiple fluorescent DNA base analogues in the same structure has been demonstrated,[11] and in none of these systems were the actual fluorophores intended to interact with DNA as nature designed. In contrast to these precedents, every base of an xDNA oligomer is inherently emissive and has the potential to change fluorescence with alterations in hybridization, sequence, and environment.

To apply the fluorescence of xDNA bases in a directed and useful manner, it is necessary to explore and characterize how the fluorescence is affected not only for the xDNA monomers, but also in their oligomeric state, and in the context of single- and double-stranded structures. Here, we report the first of these studies. We have taken a systematic approach to study short xDNA oligomers of simple sequence and have evaluated their behavior in the absence and presence of DNA complements having varied sequence and structure. We report the observation of several potentially useful fluorescence changes as a result of the interaction of xDNA bases with each other and with hybridizing DNA bases. Among the new findings are (a) long-wavelength excimer emission from adjacent xA residues, (b) enhancement of emission of $(xA)_n$ and $(xG)_n$ by hybridization opposite adenines, and (c) quenching of emission of $(xC)_n$ by hybridization opposite T or guanines. Overall, the results suggest multiple ways in which these compounds may be employed as useful reporters of structure and sequence.

Experimental Section

Nucleoside Phosphoramidite Derivatives of dxA, dxG, dxT, dxC. Syntheses of these four compounds were carried out as previously reported.[12]

Oligonucleotide Synthesis. Oligodeoxynucleotides were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer on a 1 μmol scale and possessed a 3′-phosphate group. Coupling employed standard β-cyanoethyl phosphoramidite chemistry, but with extended coupling time (600 s) for nonnatural nucleotides. All oligomers were deprotected in concentrated ammonium hydroxide (55 °C, 16 h), purified by preparative 20% denaturing polyacrylamide gel electrophoresis, and isolated by excision and extraction from the gel, followed by dialysis against water. The recovered material was quantified by absorbance at 260 nm with molar extinction coefficients determined by the nearest-neighbor method. Molar extinction coefficients for unnatural oligomers were estimated by adding the measured value of the molar extinction coefficient of the unnatural nucleoside (at 260 nm) to the calculated value for the natural DNA fragments. Previous studies have shown that xDNA bases have very low hypochromicity in xDNA oligomers.[8] Molar extinction coefficients for xDNA nucleosides used were as follows: dxA, $\epsilon_{260} = 19\,800\ M^{-1}\ cm^{-1}$; dxG, $\epsilon_{260} = 8100\ M^{-1}\ cm^{-1}$; dxT, $\epsilon_{260} = 1200\ M^{-1}\ cm^{-1}$; dxC, $\epsilon_{260} = 5800\ M^{-1}\ cm^{-1}$. Nonnatural oligomers were characterized by MALDI-TOF mass spectrometry (data are given in the Supporting Information, SI, Table S2).

Optical Methods. Steady-state fluorescence measurements were carried out at 2 μM concentration (each strand) on a Spex Fluorolog 3 fluorescence spectrometer (4-nm slit width) equipped with Lauda Brinkmann RM6 temperature controller at 25 °C. Samples were buffered at pH 7.0 with 100 mM NaCl, 10 mM $MgCl_2$, and 10 mM Na·PIPES. Buffers were not deoxygenated. Excitation wavelengths for strands containing xDNA bases were as follows: dxA and dxC, 330 nm; dxG and dxT, 320 nm. Fluorescence lifetime measurements were carried out on a PTI Easylife instrument using 325-nm LED for excitation of all xDNA bases. Fitting and analysis was performed using Felix32 software. To prevent aggregation and reabsorption of light, samples were diluted to an absorption at λ_{max} of less than 0.05.

Quantum Yields. Quantum yields of xDNA single strands were calculated in aqueous solution as reported previously[13] according to the equation:

$$\Phi_x = \Phi_R{*}(A_R/A_X){*}(E_X/E_R){*}(I_R/I_X){*}(n_X{}^2/n_R{}^2)$$

DAPI ($\lambda_{ex} = 355$ nm, $\lambda_{em} = 458$ nm, $\Phi = 0.05$ in water) was used as a reference. In all samples, total area under the curve was integrated.

Results

Sequence Design. Full exploration of xDNA fluorescence is complicated by the fact that it carries high sequence complexity; for example, the full eight-base xDNA genetic set has 512 (8^3) different trimer sequences.[6] Therefore, for this initial study we chose a simple homo-oligomeric expanded-base sequence series ($(xG)_n$, $(xA)_n$, $(xT)_n$, $(xC)_n$; Figure 1) and systematically varied the length from one to four monomers.

To investigate the effects of DNA hybridization near or opposite these xDNA bases, we conjugated the xDNA oligomers to a constant 10mer DNA oligonucleotide sequence. This allowed us to hybridize complementary DNAs of varying length and sequence near the fluorescent xDNAs and to observe any responses in emission. In each xDNA sequence, the natural base adjacent to the expanded base was chosen to be thymine, after preliminary data suggested that a single xDNA base (xT, xC) was not strongly quenched by neighboring pyrimidines.[7] The initial set of complementary DNAs was composed of 10mers complementary only to the DNA portion carrying the xDNA oligomers (with the resulting duplex having dangling xDNA bases), or 11mers or 12mers with one or two additional DNA bases placed opposite the xDNA bases. As unexpected characteristics were observed, additional complementary strands were also synthesized (vide infra).

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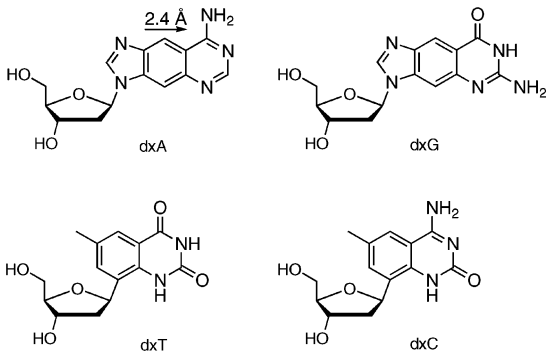
A



B

xDNA strands

5'-ATA CCA AAG T Xp-3' $(X)_1$
5'-ATA CCA AAG T XXp-3' $(X)_2$
5'-ATA CCA AAG T XXXp-3' $(X)_3$
5'-ATA CCA AAG T XXXXp-3' $(X)_4$

X = dxA, dxG, dxT, dxC

Natural DNA complement strands

5'-A CTT TGG TAT-3'
5'-Y A CTT TGG TAT-3'
5'-YY A CTT TGG TAT-3'

5' overhang region

Y = dA, dG, dT, dC

Figure 1. Nucleosides and strands used in this study. (A) The four xDNA monomers. (B) Synthesized sequences for studying effects of xDNA length and hybridization on fluorescence.

Monomer Properties. We previously reported preliminary fluorescence spectra (in methanol) for the four monomer deoxynucleosides in separate reports.[12] Note also that the xA and xG bases were previously studied as ribonucleosides by Leonard and co-workers, who first observed their fluorescence.[9a,14] For reference here, we repeated the deoxynucleoside fluorescence measurements and additionally measured excitation spectra and fluorescence lifetimes, which had not been previously reported. The four monomer emission spectra are given in Figure 2, and the optical data are presented in Table 1. Time-resolved fluorescence decay profiles are given in the SI.

Absorbance of xDNA Oligomers in Single-Stranded and Duplex Contexts. Figure 3 and Figures S1 and S2 in the SI show the absorption spectra of single strands and duplexes containing xDNA bases. Observation of the long-wavelength absorbance bands of each of the xDNA-conjugated single strands $(xN)_{1-4}$ found no significant ground-state electronic interactions between one, two, three, or four adjacent xDNA monomers, as judged by a lack of change in peak/valley shape or wavelength of the bands. Neither was there a significant interaction apparent with the adjacent thymidine, as judged by comparison of the monomer nucleosides and the DNA-conjugated monomers.

In addition, on hybridization with the control DNA complement (thus giving one through four overhanging xDNA bases at the end of a DNA duplex), there was minimal change in absorption spectra of the duplexes relative to the single-stranded cases. In this case, the xDNA oligomers are in an overhanging arrangement and are not placed opposite any DNA bases. However, they are larger than the adjacent thymine and are expected to overlap (perhaps by stacking) with the adenine in the opposite strand as well. Nevertheless, no change in absorbance is seen on addition of this complementary DNA strand for any of the 16 xDNA-containing oligomers.

Fluorescence of xDNA Oligomers ($n = 1-4$) in Single-Stranded and Duplex Contexts. Emission spectra of the single-stranded oligomers were measured using the long-wavelength absorbance maxima for excitation (Table 1). The spectra (Figure 4) showed that the xDNAs retained their monomer emission maxima in strands with one terminal xDNA and were unaffected electronically (with one exception) by the neighboring natural thymidine. Interestingly, the emission maximum of the single xG case (which is adjacent to thymine) was blue-shifted from the monomer xG nucleoside by about 20 nm (from 410 to 390 nm). Direct comparison between the strands containing a single xDNA base $(xN)_1$ showed that xA and xC appeared to have the brightest emission as judged by values of quantum yield multiplied by molar absorptivity (Table 2). With sequential addition of xDNA monomers, $(xC)_n$ and $(xT)_n$ oligomers showed no change in emission wavelength or peak shape. The $(xG)_n$ series displayed a red shift (compared with the $(xG)_1$ case), which restored the emission maximum back to that of the monomer xG nucleoside, apparently mitigating any electronic interaction with the neighboring thymine. Finally, the $(xA)_n$ series was quite different, and monomer wavelength emission was completely suppressed as a new longer-wavelength emission appeared at 520 nm (Figure 4A). This apparent excimer emission (see below) increased in intensity with the number of adjacent xA residues.

Excitation spectra for all 16 of the single-stranded oligomers are given in the SI. They revealed bands both at the long-wavelength absorption maxima and at ~260 nm, thus closely resembling the absorption spectra. Thus, the data show that the fluorescence of all xDNA bases can be elicited with excitation either at ~260 nm or at the long-wavelength peaks (320−333 nm).

Fluorescence spectra of the xDNA lone strands before and after addition of the control 10mer DNA complement showed, in general, very little effect on fluorescence emission wavelength or intensity. On average there was only slight (if any) quenching of fluorescence on hybridization (less than 15% change), indicating minimal interstrand electronic interaction between the dangling xDNAs and the opposite strand. Most notable were the simple duplexes of $(xG)_1$ and $(xG)_2$, which showed significant emission enhancements relative to the single xDNA strand (Figure 5).

Comparing the quantum yields of the monomer nucleosides (in methanol) to the monomers conjugated to the DNA (aqueous buffer), we observed that the latter values were lower, suggesting that either the buffer or (perhaps more likely) the adjacent DNA quenches the xDNA to some extent. The single xC and xT cases were quenched by about 2-fold as DNA conjugates, while xA and xG were quenched by a factor of approximately five.

To assess the degree of self-quenching occurring between the xDNA bases (if any) in oligomers of varied length, relative

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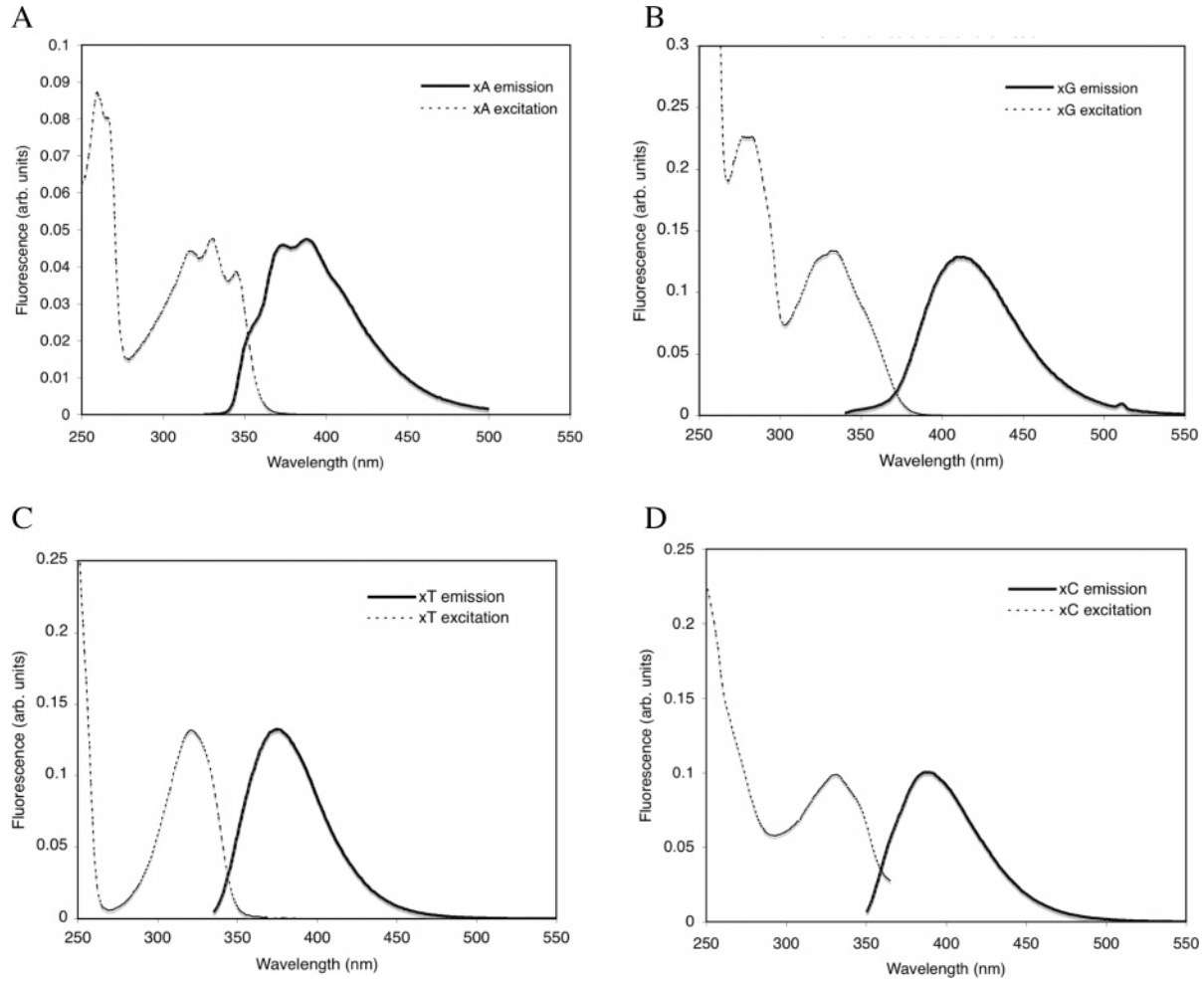

Figure 2. Normalized excitation and emission spectra for xDNA free nucleosides in methanol. (A) Excitation (dashed line, $\lambda_{em} = 393$ nm) and emission (solid line, $\lambda_{ex} = 333$ nm) spectra of dxA. (B) Excitation ($\lambda_{em} = 413$ nm) and emission ($\lambda_{ex} = 320$ nm) spectra for dxG. (C) Excitation ($\lambda_{em} = 377$ nm) and emission ($\lambda_{ex} = 321$ nm) spectra for dxT. (D) Excitation ($\lambda_{em} = 388$ nm) and emission ($\lambda_{ex} = 330$ nm) spectra for dxC.

Table 1. Summary of Photophysical Data for Free xDNA Nucleoside Monomers in MeOH

xDNA monomer	abs. λ_{max} (nm)	em. λ_{max} (nm)	$\epsilon_{\lambda max}$ (L/mol·cm)	ϵ_{260} (L/mol·cm)	Φ_{fl} (MeOH)	fluorescence lifetime (ns)	brightness[a] (L/mol·cm)
dxA	333	393	11080	19800	0.44	3.3	4900
dxG	320	413	3400	5100	0.40	7.8	1400
dxT	320	377	3400	1200	0.30	2.7	1000
dxC	330	388	4100	5800	0.52	4.0	2100

[a] Brightness calculated as $\epsilon_{\lambda max} \cdot \Phi_{fl}$.

quantum yields were determined for each single-stranded sequence (Figure 6). The quantum yield data showed that all of the homodimers of expanded bases exhibited substantial (~50%) quenching relative to their respective monomers. In addition, for three of the cases, fluorescence was further quenched, albeit slightly, on increasing the length to three or four identical xDNA bases in the sequence. However, the $(xC)_n$ series was strikingly different, as the quantum yield increased on addition of the third and fourth monomers. Very similar data were observed for the duplex series for all cases (Figure S5), again showing little effect of a neighboring complementary DNA on the optical properties of the overhanging xDNA oligomers.

Overall brightness of the single-stranded xDNA oligomers was calculated as the product of quantum yield times molar absorptivity (Table 2). The data showed that of all xDNA strands, those containing xAs or xCs were the best fluorophores, with $(xC)_4$ being the overall brightest because of a surprising increase in quantum yield relative to strands containing two and three adjacent xCs. xDNA strands composed of xGs were overall the weakest of the four, not unexpected because of lower quantum yields and molar absorptivity of xG.

Effects of Hybridizing DNA Bases opposite the xDNA Oligomers. To explore the effects of bases directly opposite xDNAs on fluorescence, next we evaluated emission of the xDNA oligomers upon hybridization with DNA complements containing one or two overhanging natural base(s) (A, T, C, G) opposite the xDNA portion. Data are shown in Figures 7 and 8 in comparison to the xDNA single-strand duplexes and with no added DNA bases opposite the xDNA. For a single base opposite the xDNAs (Figure 7), fluorescence of xDNA strands containing xA (one to four monomers) were least affected, showing virtually no change in emission. Only modest quenching by a T opposite the xA residues was observed, which was also apparent in strands containing xG. In the case of xC and xT, however, quenching by a single T base was pronounced, with 2−4-fold decrease in emission observed in $(xC)_n$-containing duplexes as compared to the case with no DNA base opposite (or as compared with the xDNA-containing single strand alone). As for cases with a single C, G, or A in the complement strand

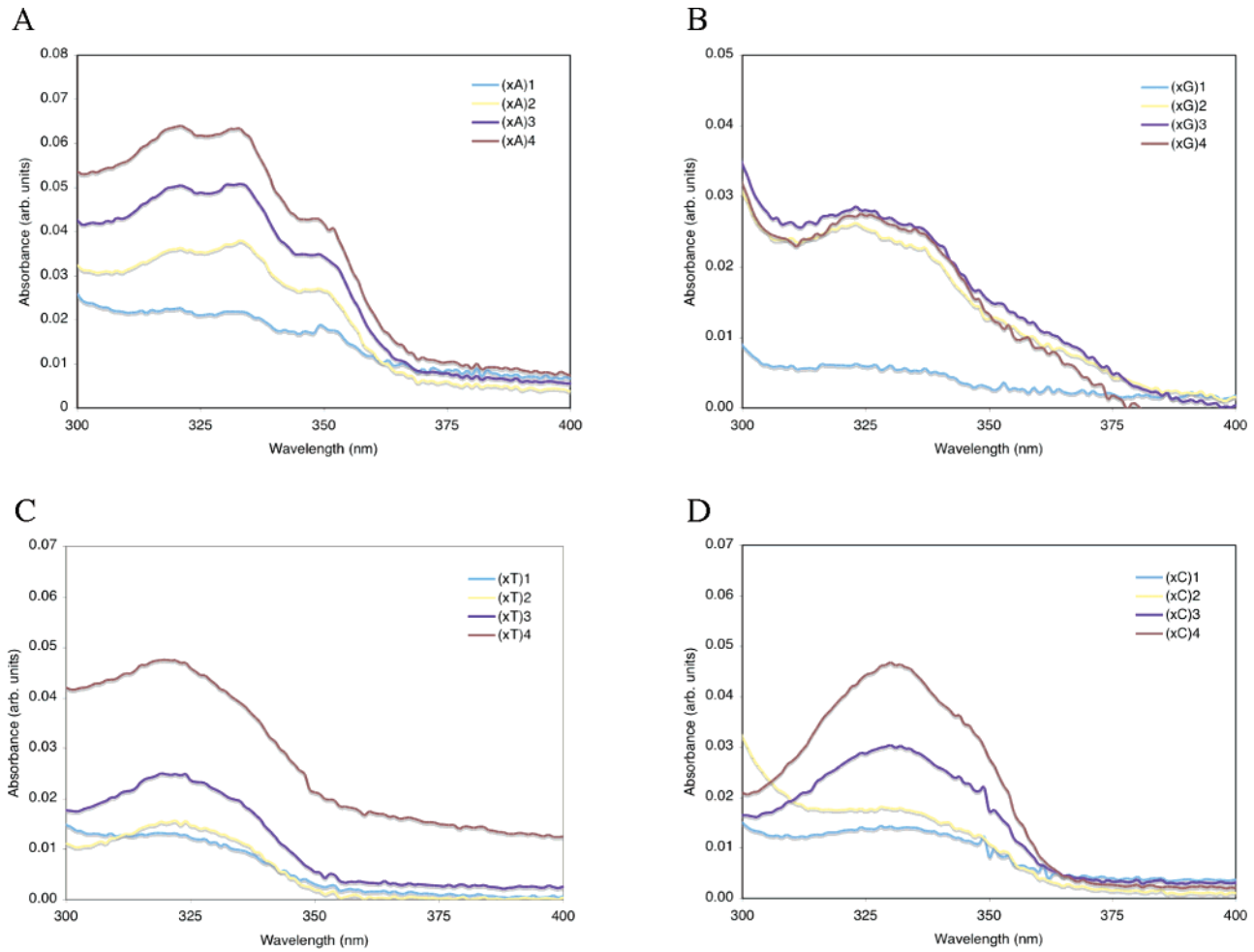

Figure 3. Absorption spectra of xDNA-containing oligomers used in this study. Sequences contain one, two, three, and four of the same xDNA base on the single-stranded 3′ end, all at equimolar concentration. Data for >300 nm are shown; see SI for full spectra. (A) Spectra of single strands with one to four xA monomers. (B) Spectra of single strands with one to four xG monomers. (C) Spectra of single strands with one to four xT monomers. (D) Spectra of single strands with one to four xC monomers. Measurements were made in aqueous buffer (100 mM Na^+, 10 mM Mg^{2+}, 10 mM Na·PIPES, pH 7.0).

opposite the xDNAs, fluorescence changes were generally small or zero.

When two identical natural DNA bases were in the complement strand opposite the xDNAs, the effects on xDNA base fluorescence were greater. Figure 8 shows the emission spectra for a representative subset; full data are given in Figures S10−13 in the SI. In the $(xG)_n$ series, two adenines placed opposite resulted in enhancement and red-shifting of $(xG)_2$. In the $(xA)_n$ series, two adenines enhanced and red-shifted the monomer fluorescence band at 393 nm, but had no effect on the excimer band of the longer $(xA)_{3,4}$ cases. The xDNA pyrimidine responses were different: the main response was quenching caused by addition of two G bases opposite either $(xC)_n$ or $(xT)_n$.

Polyadenine Enhancement Effect. The above data revealed two substantial and strikingly opposite classes of response of xDNAs to addition of DNAs directly opposite them in a duplex: the first was enhancement of fluorescence of the expanded bases by one or more adenines, and the second was quenching of the expanded pyrimidines by one or more guanines. We therefore synthesized additional DNAs to further explore these special cases. First we addressed the polyadenine fluorescence enhancement, to determine whether fluorescence emission would be enhanced further by additional A's opposite the xDNA residues.

Two new complementary strands containing four and six adjacent adenines in the 5′ overhang region were synthesized and used in hybridization experiments as before. Figure 9 shows representative results with the whole polyadenine series (see Figures S14−S17 for the complete data set). In the $(xC)_{2-4}$- and $(xT)_{2-4}$-conjugated strands, fluorescence emission increased yet further on hybridization with complements containing four overhanging adenines relative to those with two; the maximum enhancement on hybridization was ca. 75% with $(xT)_2$. However, the cases with six A residues surprisingly resulted in the loss of emission enhancement. As for the $(xG)_n$ strands, both A_2 and A_4 showed the strongest enhancements of all the xDNAs, with up to a 2−3-fold increase upon hybridization. Interestingly, a further extension to A_6 resulted (as with the expanded pyrimidines) in a loss of the effect. Finally, the $(xA)_n$ series showed only slight enhancements in selected cases with A_2 and A_4.

Polyguanine Quenching Effect. To explore further the effects of guanines opposite the fluorescent xDNA oligomers, we prepared additional DNA complement strands containing three, four, six, seven, eight, and nine consecutive guanines at their 5′ termini. Representative results are shown in Figure 10 (see also SI Figures S18−S21). Results of hybridization experiments showed that, in some cases, strong quenching

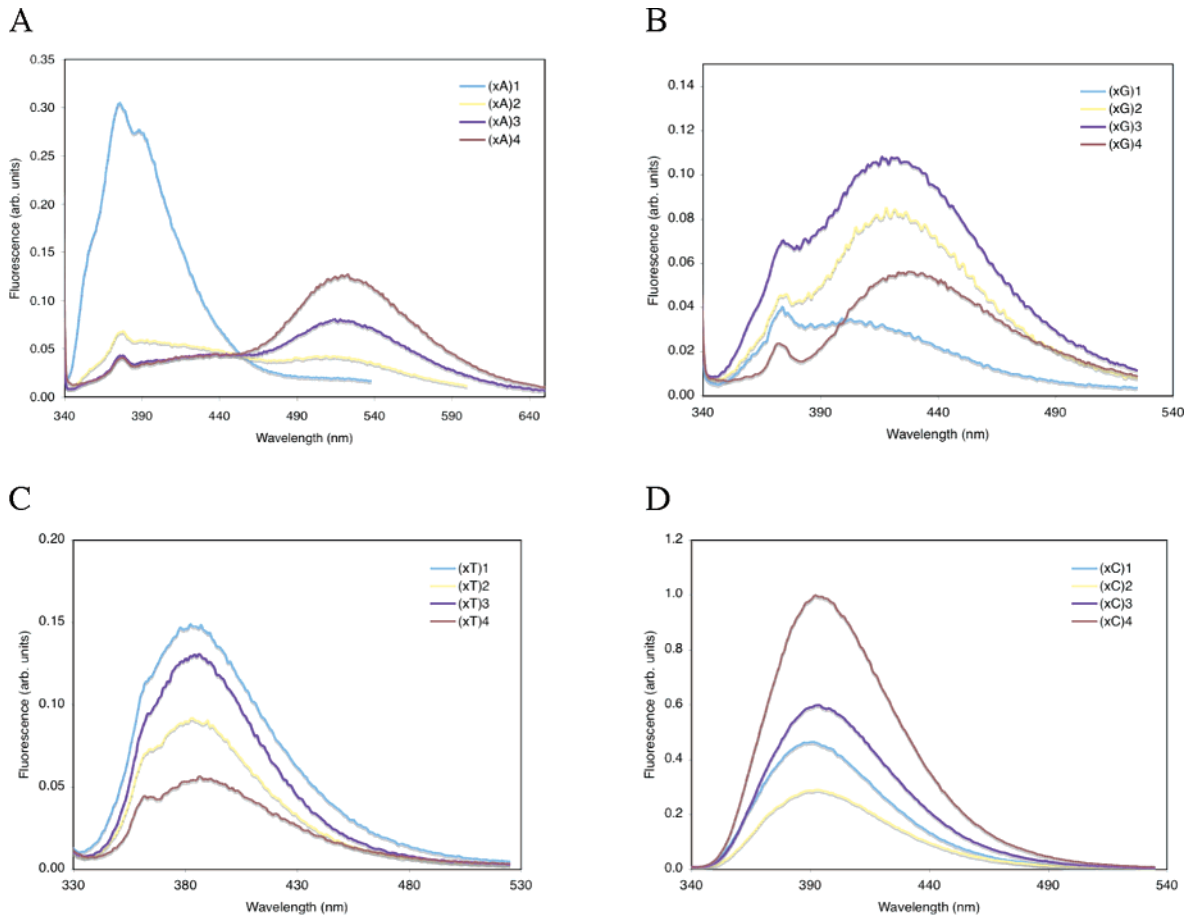

Figure 4. Emission spectra of the single strands in this study. (A) Emission spectra of $(xA)_n$ strands (excitation 333 nm). (B) Emission spectra of $(xG)_n$ strands (excitation 320 nm). (C) Emission spectra of $(xT)_n$ strands (excitation 321 nm). (D) Emission spectra of $(xC)_n$ strands (excitation at 330 nm). See Figure 3 for conditions.

Table 2. Relative Brightness[a] of xDNA Oligomers in This Study

$(xA)_n$	brightness	$(xG)_n$	brightness	$(xT)_n$	brightness	$(xC)_n$	brightness
$(xA)_1$	890	$(xG)_1$	270	$(xT)_1$	480	$(xC)_1$	1300
$(xA)_2$	660	$(xG)_2$	270	$(xT)_2$	480	$(xC)_2$	1300
$(xA)_3$	660	$(xG)_3$	610	$(xT)_3$	610	$(xC)_3$	2300
$(xA)_4$	890	$(xG)_4$	410	$(xT)_4$	270	$(xC)_4$	3400

[a] Brightness calculated as $\epsilon_{\lambda max} \cdot \Phi_{fl}$.

occurred, and in nearly all cases, the degree of quenching correlated well with the number of guanines on the complement strand. The $(xC)_n$ oligomers were quenched to the greatest extent, exhibiting up to 95% quenching for a complement with six consecutive guanines in the overhang. The $(xT)_n$ oligomers were second most strongly quenched, and in both $(xC)_3$ and $(xT)_3$ strands, the cases with three consecutive expanded bases were quenched the most strongly (95 and 65%, respectively). Hybridization with complements containing seven, eight, and nine consecutive guanines did not result in further increases in quenching, as some emission from $(xC)_3$ and $(xT)_3$ was restored in these duplexes (Figure S22). As was observed with the two-base DNA complements, the $(xA)_n$ and $(xG)_n$ cases showed no significant quenching by multiple guanines (Figures S20 and S21).

Discussion

The above data yield basic information both on oligomeric xDNAs and on their monomer components. First to be noted

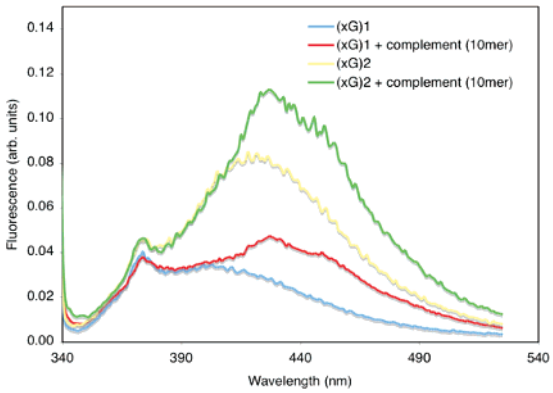

Figure 5. Emission spectra (excitation 320 nm) of xDNA-containing strands containing one (blue) and two (yellow) dxGs, and their change in emission on duplex formation with a complementary 10mer (red, green, respectively). All samples 2 μM (each strand) in the buffer described in Figure 3.

are the fluorescence properties of the individual xDNA bases, which could be useful in applying them as tools in biochemical and biophysical studies. As general fluorophores, their high quantum yields and moderate molar absorptivities compare reasonably well with common blue fluorophores: for example, dansyl ($\Phi_{em} = 0.03$, $\epsilon = 5300\ M^{-1}\ cm^{-1}$)[15] and DAPI ($\Phi_{em} = 0.04$, $\epsilon = 27\ 000\ M^{-1}\ cm^{-1}$).[16] However, the xDNA monomers

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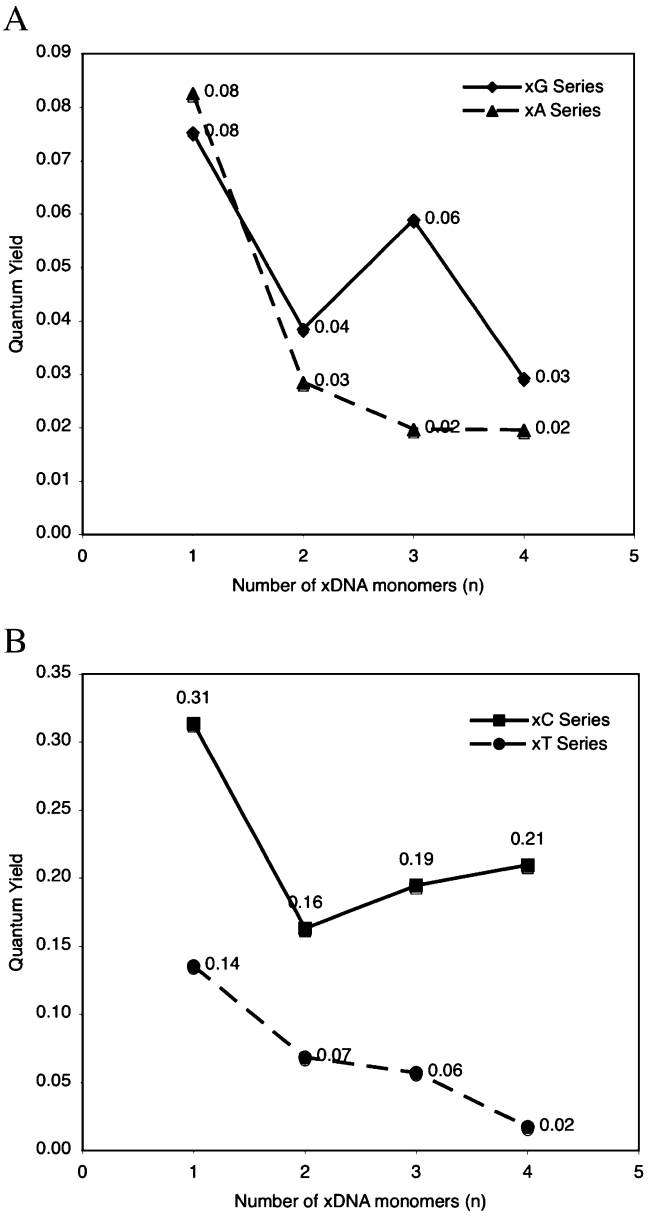

Figure 6. Trends in quantum yields of xDNA single strands used in this study as adjacent xDNA bases are added at the 3′ end. (A) Data for oligomers containing the expanded purines $(xG)_n$ (solid line) and $(xA)_n$ (dashed line). (B) Data for oligomers containing the expanded pyrimidines $(xC)_n$ (solid line) and $(xT)_n$ (dashed line). See Experimental Section for methods and conditions.

are distinct from most fluorophores by being nucleobase analogues, and there are relatively few of those available for comparison. 2-Aminopurine (2AP) ($\Phi_{em} = 0.68$, $\epsilon = 6000\ M^{-1}\ cm^{-1}$),[17] coumarin deoxyriboside ($\Phi_{em} = 0.65$, $\epsilon = 23\ 500\ M^{-1}\ cm^{-1}$),[16,18] tC deoxycytosine analogue ($\Phi_{em} = 0.20$, $\epsilon = 4500\ M^{-1}\ cm^{-1}$),[19] and pyrene deoxynucleoside ($\Phi_{em} = 0.12$, $\epsilon = 47\ 000\ M^{-1}\ cm^{-1}$)[11c] are among the most prominent examples. Like many nucleoside analogues, the xDNA bases can be quenched to some extent by neighboring DNA bases; this is also true of most of the above dyes except tC.[3d] This quenching has been useful for 2AP and pyrene deoxyriboside, because such context-dependent dyes can report on changes in structure of DNA adjacent to the fluorophore. Further experiments will be needed to evaluate how the xDNA bases are affected by other neighboring DNA bases besides thymine. Some xDNA bases, such as xA, are robust fluorescence emitters even inside the double helix. This unique property coupled with xA's defined geometry in DNA are potentially useful in probing DNA structure via FRET or anisotropy.[10a,20] Finally, like 2AP and tC analogues, the xDNA bases retain the ability to recognize complementary DNA bases,[6−8,12b] and thus there are likely to be biophysical applications where they may prove useful in contexts where the added size is not problematic. In some applications, large-sized nucleobases have in fact proven advantageous; for example, pyrene has been used to "flip out" complementary bases in studies of DNA repair enzymes.[21] In addition, it has been shown that a strand composed of all xDNAs can bind a natural complement with high affinity.[6] In conjunction with the results reported above, it is feasible that a simple strand of all inherently fluorescent xDNA bases could be used to detect mismatched DNA. Future studies will be directed toward this possibility.

Our experiments show that positioning successive xDNA bases together changes their emission properties in varied ways depending on their length. The data establish that $(xT)_n$ and $(xG)_n$ oligomeric strands of xDNA bases retain their fluorescence with moderate self-quenching. Interestingly, the $(xC)_n$ case behaves differently, increasing overall intensity with greater length, with both quantum yield and light-gathering ability rising over the series. Finally, the $(xA)_n$ cases behave the most distinctly, yielding a long-wavelength (520 nm) emission with two or more monomers. This is characteristic of excited-state dimer (excimer) emission[22] and closely resembles the behavior seen previously for the fluorescent pyrene deoxyriboside.[23] Recent studies have made much use of the pyrene monomer/excimer dual behavior in genetic reporting;[24] for example, adjacent binding of two pyrene-containing probes on a genetic target can yield distinct excimer emission.[25] The current results, in conjunction with xA's capacity to retain fluorescence on hybridization, suggest that xA may potentially be useful in similar types of applications; the ability of xA to retain Watson−Crick-like recognition may add further capabilities as well.

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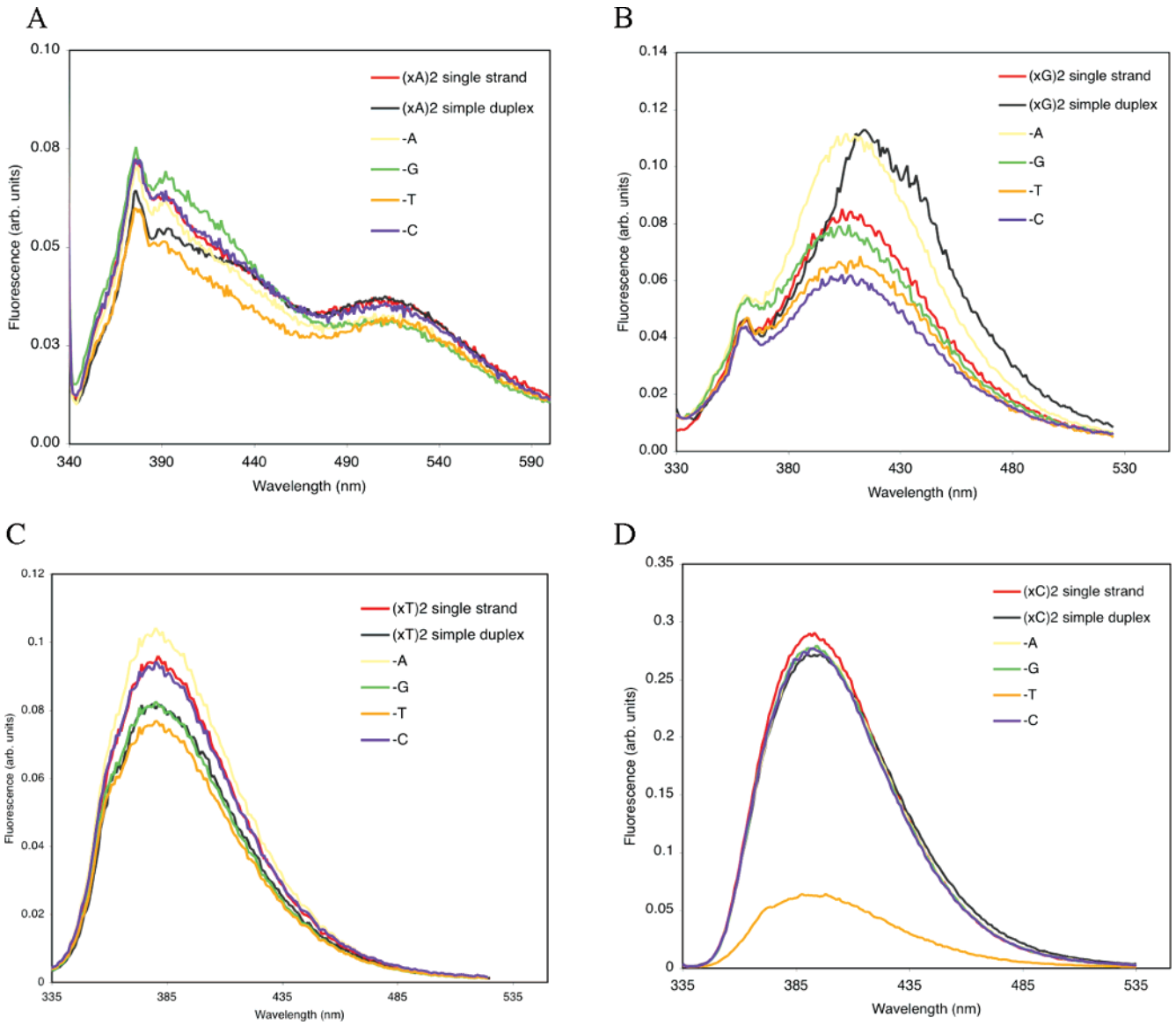

Figure 7. Fluorescence emission spectra of duplexes with a single DNA base opposite the xDNA domains. The DNA complements contain either zero (black line) or one A, G, T, C (yellow, green, orange, violet, respectively) in the overhang region opposite the xDNA residues. In each case, fluorescence of the single xDNA strand is shown in red for comparison. (A) Spectra of $(xA)_2$-containing duplexes (excitation 333 nm). (B) Spectra of $(xG)_2$-containing duplexes (excitation 320 nm). (C) Spectra of $(xT)_2$-containing duplexes (excitation 321 nm). (D) Spectra of $(xC)_2$-containing duplexes (excitation 330 nm). See Figure 5 for conditions. See Figures S6−S9 for the full data set.

The ability of xDNAs to retain, lose, or enhance fluorescence on binding a complementary strand could prove useful in the realm of probes for hybridization or DNA structure. Because xDNA sequences are complex,[5b,6] we limited the sequences to short xDNA homo-oligomers for this initial study. Our results show widely varied effects of hybridizing natural bases opposite the xDNA residues, from no effect, to substantial enhancement of emission, to strong quenching. The data suggest that in complex xDNA sequences it may be difficult (at least until more studies are available) to predict the fluorescence changes that occur on hybridization. However, the results do show clear and reliable changes for simple sequences, which suggest immediate applications in probes where sequence choice is flexible, such as in overhanging ends and in stems of stem−loop-type probes.

Note that in the present context we make no general assumptions about whether the xDNA residues at the end of a 10mer DNA will form base pairs or not with DNA bases placed opposite them in a complementary strand. There are cases in which it is reasonable to speculate that specific pairing may form, however,[26] and this pairing may translate to the unique response observed. For example, with three xC residues at the end of the DNA duplex opposite three (or more) guanines it is very possible (if not likely) that three xDNA base pairs are formed. Thermal denaturation data (SI) illustrate a marked stabilization of the duplex on adding one G to three G's opposite the $(xC)_3$ oligomer (35, 50, 57 °C, respectively) with stability leveling off on further addition of overhanging G's. This suggests additional stabilization by Watson−Crick-like base pair formation in the xC-G examples. In contrast, in the $(xT)_3$ oligomer, which would not be expected to fully pair with G, stabilization is not as pronounced (35, 35, 38 °C, with placement opposite one, two, three G residues, respectively). It is possible that the $(xT)_3$ oligomer forms a wobble geometry with G bases, which may explain the slight stabilization observed. In addition,

(26) Liu, H.; Lynch, S. R.; Kool, E. T. *J. Am. Chem. Soc.* **2004**, *126*, 6900−6905.

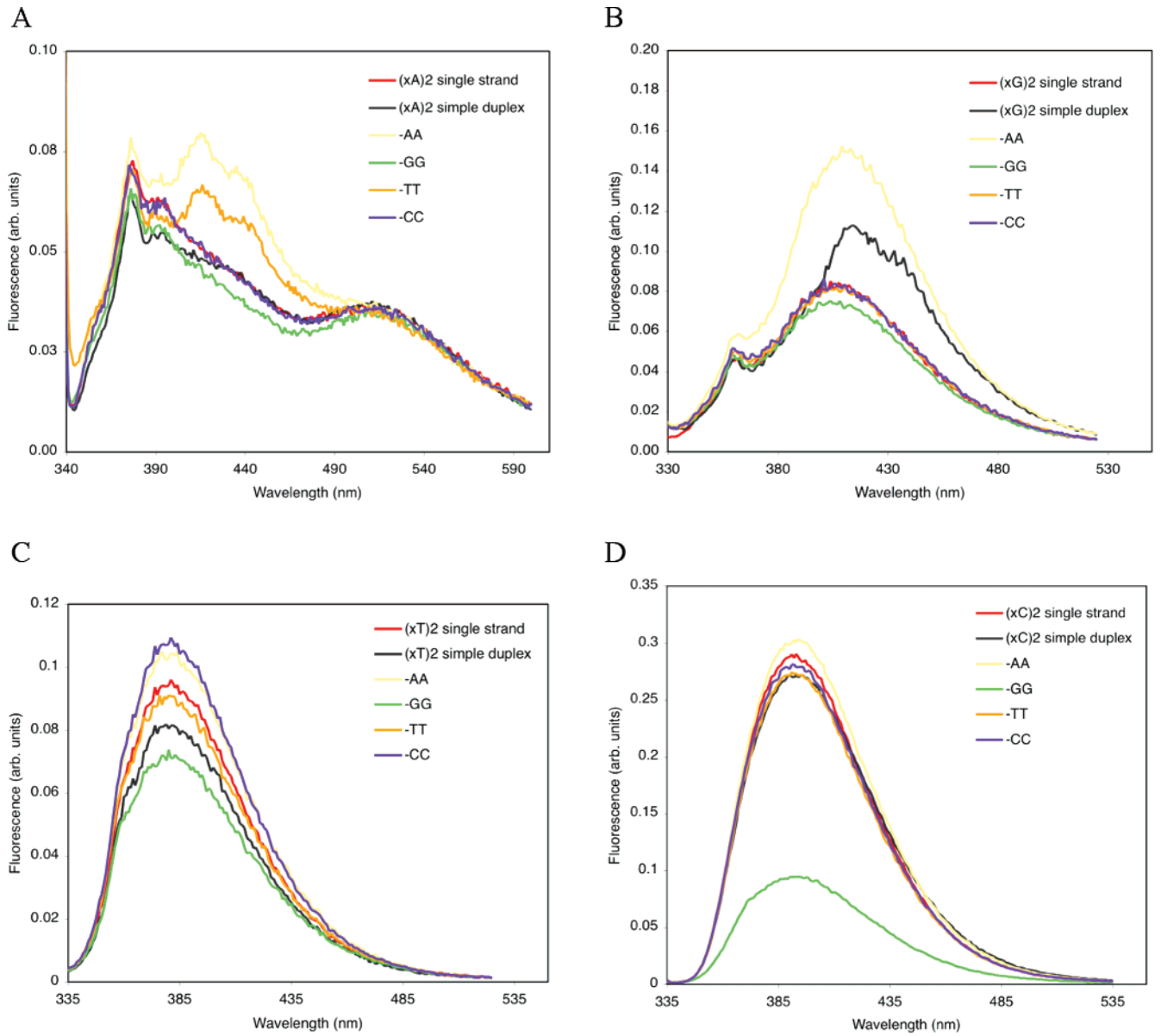

Figure 8. Fluorescence emission spectra of xDNA-conjugated strands containing two identical 3′ xDNA residues hybridized with complements containing two adjacent A, G, T, or C's (yellow, green, orange, violet, respectively) opposite the xDNA. In each case, fluorescence of the single xDNA strand is shown in red for comparison, and the black line shows the complement lacking any bases opposite the xDNA. (A) Data for $(xA)_2$. (B) Data for $(xG)_2$. (C) Data for $(xT)_2$. (D) Data for $(xC)_2$. Conditions are the same as those in Figure 7. See SI for full data set.

previous data with single xDNA bases in the middle of otherwise all-DNA duplexes suggest that the single xDNA pairs do form, although the DNA-xDNA junction appears to be destabilizing as a result of the difference in size.[7] However, addition of up to three xDNA base pairs at this junction actually resulted in slight stabilization in that previous report, which may better explain the present cases with multiple xDNAs opposite multiple DNAs. In the current experiments with mismatched cases (such as $(xT)_3$ opposite G_3), multiple structural arrangements are possible, including wobble-type duplex, some form of mutually intercalated structure, or no well-defined structure at all. Thus, conclusions from the present data are best limited to geometries at the ends of duplexes. Future experiments will be directed at fluorescence behavior in other structural arrangements of xDNA bases.

Although quenching of some of the xDNAs by nearby DNA was an expected outcome, it was surprising to observe that, for some specific cases, fluorescence emission actually increases on duplex formation, and in some cases quite substantially. For example, xG strands showed a 2−3-fold increase in emission when hybridized in a duplex opposite multiple adenines. It is interesting that the effect is most pronounced with $(xG)_n$ but is weak with $(xT)_n$ and $(xC)_n$ and is absent for $(xA)_n$ cases. The reason for this enhancement is not yet clear. It is possible that the unfavorable interaction of the larger purine bases in the complementary strand causes the xDNA bases to distort, which may mitigate some self-quenching. Thermal denaturation studies with the $(xG)_3$-substituted oligomer (SI) showed that increasing numbers of adenines on the opposing strand opposite $(xG)_3$ did not cause significant duplex stabilization (34, 35, 34 °C for one, two, and four A's, respectively). In contrast, when $(xG)_3$ was paired with complements containing multiple G's (which did not cause emission enhancement on hybridization), marked stabilization was observed (34, 40, 48 °C for one, two, and four

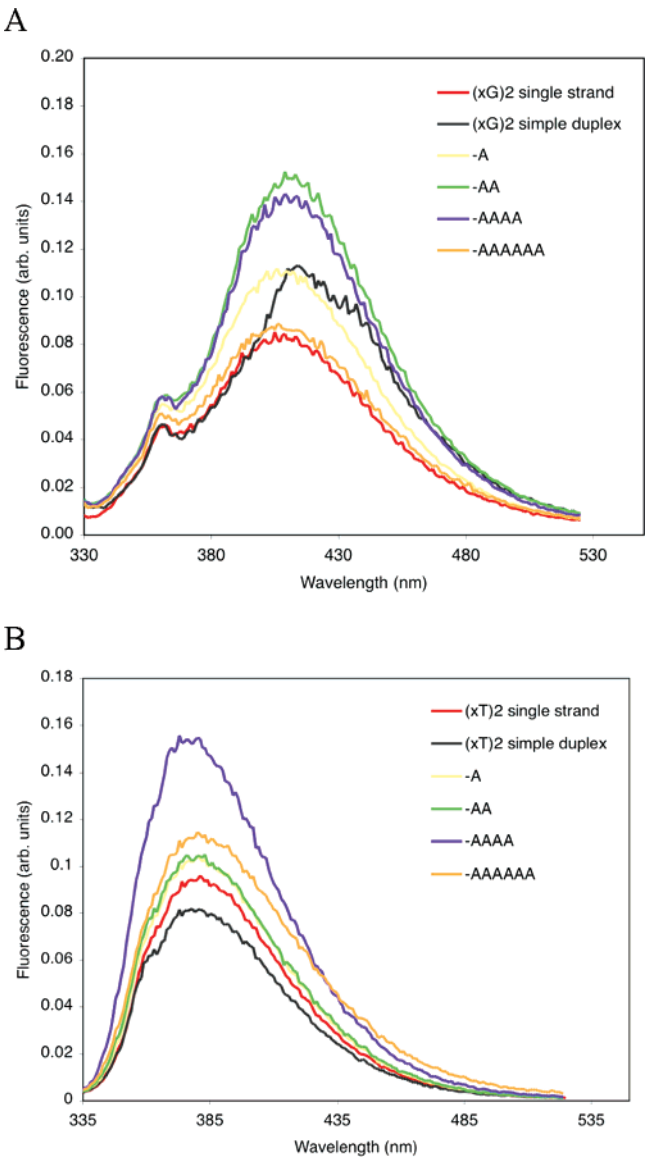

Figure 9. Fluorescence enhancement of xDNA bases by natural adenines placed opposite. Data show emission spectra of xDNA-conjugated strands containing two xGs or xTs hybridized with complements having up to six adenines. (A) Data for $(xG)_2$-substituted cases. (B) Data for $(xT)_2$-substituted cases. (Data for all cases shown in the SI.) See Figure 8 for conditions.

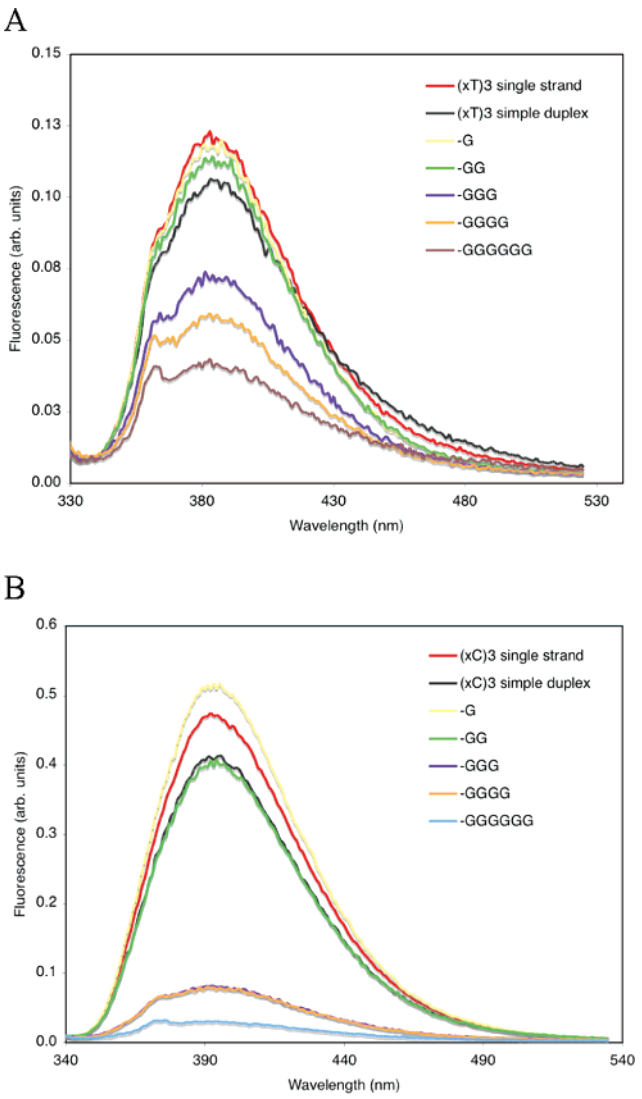

Figure 10. Fluorescence quenching of xDNA bases by natural guanines placed opposite. Data show emission spectra of xDNA-conjugated strands containing three xTs or xCs hybridized with complements having up to six guanines. (A) Data for $(xT)_3$-substituted cases. (B) Data for $(xC)_3$-substituted cases. (Data for all cases shown in the SI.) See Figure 8 for conditions.

G's, respectively). This may suggest less helical distortion (which might lead to emission enhancement) in the $(xG)_3$-G_n cases. Structural studies would be useful in probing this possibility. Regardless of the mechanism, one can imagine probe designs that take advantage of this enhancement for reporting on specific nucleic acid structures or sequences (for example, polyadenine sequences) and the selective response of some xDNA bases with each of the natural DNA bases.

Our experiments also reveal a second (and opposite) strong change in xDNA fluorescence upon hybridization; namely, the quenching of expanded pyrimidines by multiple G bases. The degree of quenching we observe is substantial (up to 95%), reaching levels of quenching commonly observed with commercial dye-quencher pairs.[27] The photophysical and structural reasons for this quenching are as yet unclear, although quenching of fluorescence by guanine is not unexpected. Guanine is most easily oxidized of the natural bases and has been shown to undergo photoinduced electron transfer (PET) to other fluorophores, especially GG doublets and poly G sequences.[27,28] Recent theoretical calculations of xDNAs have provided HOMO−LUMO energies for all expanded bases,[29] which corroborate that xC and xT are most likely to undergo PET from guanine. Overall, the reason for most favorable quenching of xC by guanines may be due to either favorable photophysical interactions, or to xDNA base pair formation, or both. Recent literature has shown that excited states in natural guanine are much shorter lived when it is Watson−Crick hydrogen-bonded to cytidine, relative to nonpairing.[30] This possibility may give xDNA

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advantages over other base analogues not capable of pairing with the natural genetic system.

The expanded pyrimidine oligomers were selectively quenched by a single opposing thymidine. Quenching of $(xC)_2$ was most dramatic (~75%) of all the series, although all $(xC)_n$ cases were quenched (Figure S9). The strong fluorescence quenching ability of thymine has been observed with other fluorophores such as pyrene[32] and has been attributed to PET from the excited-state fluorophore to thymine, the mostly readily reduced DNA base. Given the similar electronics of xC and xT,[29] it seems reasonable that the expanded pyrimidines are affected differently than the xA and xG oligomers.

The quenching experiments are useful in delineating a strong quenching response, since equilibria between quenched and emissive states have been widely applied recently in sequence-based probes such as molecular beacons.[31] Indeed, the fact that the present quenching occurs from the DNA residues (as opposed to a conjugated explicit quencher molecule) has some potential advantages. For example, recent studies have made use of quenching of pyrene by thymine to construct "quencher-less" designs of beacon-type probes.[32] In the present case, xDNA molecules have the added advantage of requiring no separate fluorophore either, and thus these experiments suggest some particularly efficient and compact probe designs that make use of (a) the inherent fluorescence of xDNA, (b) the internal quenching by guanines, and (c) the high stability and selectivity of xDNA helices. Future studies will be directed at exploring such designs.

Overall, our data show clearly that, in some cases (such as with xC-G pairing and with polyadenine targets), it is possible for size-expanded DNA bases and oligomers to selectively recognize and report on DNA sequences and structures via their built-in fluorescence. The ability of the four expanded bases of xDNA to interact favorably with the natural genetic system as well as report on this interaction may confer multiple uses of this unusual genetic set in the arena of nucleic acid detection and reporting/probing of biological structures and events.

Acknowledgment. We thank the National Institutes of Health (GM63587) for support.

Supporting Information Available: Absorption, emission, and excitation spectra of all duplexes; fluorescence lifetime data; mass spectrometric data for xDNA-containing oligonucleotides; and thermal melting data. This material is available free of charge via the Internet at http://pubs.acs.org.

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