A Sequence-Specific Threading Tetraintercalator with an Extremely Slow Dissociation Rate Constant

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Abstract

A long-lived and sequence specific ligand-DNA complex would make possible the modulation of biological processes for extended periods. We have been investigating the threading polyintercalation approach to DNA recognition in which chains of aromatic units thread back and forth repeatedly through the double helix. Here we report the preliminary sequence specificity and detailed kinetic analysis of a structurally characterized threading tetraintercalator. Specific binding on a relatively long DNA strand was observed, strongly favoring a predicted 14-base pair sequence. Kinetic studies revealed a multi-step association process and specificity was found to derive primarily from large differences in dissociation rates. Importantly, the rate-limiting dissociation rate constant of the tetraintercalator complex dissociating from its preferred binding site was extremely slow, corresponding to a 16 day half-life, making it one of the longer non-covalent complex half-lives yet measured, and, to the best of our knowledge, the longest for a DNA binding molecule.

Molecules that bind DNA can modulate transcription, repair, and replication both *in vitro* and *in vivo*, so targeting specific sequences of DNA with small molecules has been a field of continued interest for over 30 years. For example, Dervan and co-workers have developed a series of polyamide molecules that bind in dimeric fashion to DNA with programmable

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specificity via the minor groove,[1,2] with a current focus on understanding the rules for delivery and distribution *in vivo*.[3,4] Other modular approaches include triple-helix forming oligonucleotides and peptide-nucleic acids (PNAs).[5-8]

For possible *in vivo* studies in the laboratory, as well as looking ahead to potential therapeutic applications for addressing long-term chronic diseases, it is of interest to develop DNA binding molecules exhibiting very long-lived complexes, capable of modulating biological processes on timescales relevant to organism lifetimes. With this in mind, we have been investigating threading polyintercalation, a binding mode that places a significant number of functional groups in both DNA grooves. For example, by linking the naphthalene diimide (NDI) units, known threading intercalators,[9,10] in a head-to-tail fashion with a variety of flexible peptide linkers, a growing family of unique DNA-binding molecules has been developed.[11-17] Threading *bis*-intercalators **1** and **2** were previously identified through library screening experiments in which the peptide linkers were randomized.[12] Structural characterization of the complexes with their respective preferred DNA binding sites revealed that sequence specific recognition was taking place with linkers spanning four base pairs (bp) through the major and minor grooves of DNA for **1** and **2**, respectively (Fig. 1a & 1b).[12-14] This work complements studies with a variety of other synthetic and naturally occurring mono- and bis- DNA intercalators.[10, 18-24]

Tetraintercalator **3** was designed with linkers intended to bind with a minor–major–minor groove topology (Fig. 1c). The two β-alanyl-β-alanyl-β-alanyl-*l*-lysine (-βAla$_3$-Lys-) minor groove binding linkers were taken from **2**. Recall that β-alanine is the biochemical name often used for 3-aminopropanoic acid. Adipic acid with two *l*-lysine residues attached was chosen to be the central major groove binding linker because modeling indicated this linker would reproduce many of the structural features of the glycyl-glycyl-glycyl-*l*-lysine linker of **1**, while offering the advantage of introducing C_2 symmetry into **3,** simplifying NMR spectra and thus structural analyses. Consistent with the original design, a detailed NMR structural characterization verified threading polyintercalation, and revealed the linkers to be distributed in the expected minor-major-minor groove topology (Fig. 1c).[15]

An anticipated consequence of the threading polyintercalator design of **3** is an extremely slow dissociation from its preferred DNA sequence. This expectation is based on the observation that for one of the two internal NDI units to dissociate, the adjacent terminal NDI must dissociate first. For the entire molecule to dissociate, the other two NDI units would have to also dissociate in a coordinated fashion before re-binding occurs. The unlikely nature of this highly concerted set of motions should reduce the dissociation rate significantly. One might expect the association rate to be proportionately slow due to the complicated nature of the bound topology. In addition to kinetics, the overall sequence specificity of the interaction has not previously been addressed with a threading tetraintercalator such as **3**.

Herein, footprinting studies verified sequence specific binding by **3** in the context of a relatively long piece of DNA that contained a large number of other possible binding sites. Additionally, the detailed kinetic analysis of **3** binding to oligonucleotides with and without its preferred DNA sequence were measured, revealing a multi-step association as well as a

sequence specific and remarkably slow dissociation process. We report the slowest dissociation half-life of any non-covalent interaction with DNA, which could ultimately lead to prolonged disruption of transcription or other biological processes.

Results

DNase I footprinting

DNase I footprinting was used to investigate the ability of **3** to bind its 14 bp preferred site within the context of a 467 bp DNA fragment. A clear footprint for **3** binding selectively to sequence A with high specificity was observed at 63 nM after 48 hr (Fig. 2). Non-specific binding was not seen, even at 500 nM, verifying the highly specific and predictable nature of DNA binding by **3**. It is assumed that **3** is binding to sequence A with the same threading topology observed in the NMR structural analysis reported previously for this sequence, although there is formally no direct experimental evidence to support this assumption on long pieces of DNA.[15] Quantitative footprinting of binding thermodynamics was precluded by the extensive time needed to approach equilibrium at concentrations near our estimated K_D value[25] and the limited dynamic range of the footprinting method at the low concentrations of labeled DNA required.

Dissociation kinetics

A ^{32}P labeled oligonucleotide containing the 14 bp tetraintercalator binding site (**oligo A**, Fig. 3a) was incubated with a stoichiometric amount of **3**, followed by separation using native polyacrylamide gel electrophoresis. The **3** - **oligo A** complex exhibited retarded migration on the gel. A control oligonucleotide (**oligo B**, Fig. 3a) containing a randomly chosen sequence was also incubated with **3**, but did not produce an observable gel shift.

Gel shift assays were used to determine the dissociation rate constant for **3** from **oligo A** containing the preferred binding site. In these experiments a 100-fold excess of unlabeled **oligo A** was added to a solution containing the ^{32}P-labeled **oligo A** occupied by a stoichiometric amount of bound **3** (Fig. 3b). We assume that this technique measures the rate-limiting dissociation rate constant as **3** leaves its binding site. Gel shifts could be analyzed up to 34 days before loss of radioactivity reduced the signal-to-noise ratio and thus the dynamic range of the assay below useful levels. The data was fit to a standard monoexponential decay equation (Fig. S1, Supplementary Information) revealing a dissociation rate constant (k_d) of $5.0 \pm 0.5 \times 10^{-7}$ s^{-1} at 100 mM NaCl, which corresponds to a half-life of 16 days (Table 1). The observed k_d increased with increasing NaCl concentrations over the range 25 mM to 250 mM, which is not surprising given the four *l*-lysine residues within **3** that presumably make electrostatic contacts with the phosphodiester backbone of DNA. A plot of log k_d as a function of $-\log [Na^+]$ was linear with a slope of 0.59 (Fig. S2, Supplementary Information).

Nonspecific dissociation kinetics

In order to quantify the sequence specific behavior of **3**, the dissociation rate constant from a randomly chosen sequence, **oligo B**, was also determined. As mentioned previously, gel electrophoresis could not be used to monitor the dissociation because a gel shift could not be

detected for the **3-oligo B** complex, presumably due to rapid dissociation. Instead, the dissociation of **3** from **oligo B** was monitored using stopped-flow methods in the presence of 2% sodium dodecyl sulfate (SDS) monitored for 200 seconds (Fig. 3c).[18,26] The absorbance trace was fit to a double exponential function, suggesting a two-step mechanism, with the observed first-order dissociation rate constants of the fast and slow steps, k_1 and k_2, determined to be 0.29 s^{-1} and 0.019 s^{-1}, respectively, at 100 mM NaCl. Note that SDS has been shown to enhance the dissociation rate for cationic intercalators that bind DNA, so values reported here should be considered upper-limit estimates.[27] Using the slower dissociation step for comparison, these results demonstrate that the dissociation of **3** from **oligo A** containing the preferred binding site is roughly 4×10^4 times slower compared to **oligo B**, verifying that the differences in kinetic off-rates is largely, if not exclusively, responsible for sequence selectivity. Salt dependence of the dissociation rate was also examined for the **3-oligo B** complex (Table 1), and the log k_d versus –log [Na$^+$] plot was linear with a slope of 0.54 (Fig. S3, Supplementary Information).

Association rate measurements

To study the association rates of **3**, a multi-pronged approach was pursued: 1D ^{1}H NMR, gel mobility-shift assay, and stopped-flow UV-visible spectroscopy.

1D ^{1}H NMR studies were performed using the same 14 bp binding site oligonucleotide (**oligo C**, Fig. 3a) used previously,[15] at a final concentration of 60 μM mixed directly in an NMR tube with a stoichiometric amount of **3**. After a total of 5.6 minutes to mix and scan the sample, analysis of the spectra revealed that all signals were identical to a fully equilibrated sample (Fig. 4).[15] In other words, **3** was fully intercalated in the **oligo C** binding site with the linker in the expected minor-major-minor groove topology after 5.6 min. The **3-oligo C** NMR sample was subsequently monitored at different time points over a 24 hr period (Fig. S4, Supplementary Information) with no observable change.

As a qualitative approach to monitoring association, the gel shift assay was used with stoichiometric amounts of **3** and **oligo A** in the concentration range of 250 nM to 2,500 nM. These experiments were used to gain a rough estimate of an association rate constant to be compared with, and inform, the more accurate stopped-flow spectroscopic measurements described below. Using the integrated rate equation for stoichiometric binding[28] (Equation (1), Supplementary Information) yielded an estimated association rate constant of $1 \pm 0.5 \times 10^4$ M^{-1} s^{-1}. The advantage of the gel shift approach is that we already know that non-specific association does not produce a gel shift with **3**. Thus, the estimated association rate constant measured in this way is expected to represent sequence specific binding.

The rates of association between **3** and either **oligo A** or **oligo B** were also analyzed using stopped-flow methods. Hypochromism accompanies intercalation, so the time-dependent decrease at the visible absorbance maximum (386 nm) of **3** was monitored following the mixing of equal volumes of solutions containing **3** or DNA. Absorbance traces were collected for fixed concentrations of **3** mixed with increasing DNA concentrations such that the final ratios after mixing ranged from 1:1 to 1:8. An initial rapid decrease in absorbance was observed, reflecting a fast association of **3** with DNA (Fig. 5a).

Single or two-step models did not fit the data satisfactorily, therefore kinetic traces obtained at each DNA concentration were fit globally[29] to a minimal three-step irreversible mechanism (Fig. 5b). The model assumes an initial fast interaction followed by slower consecutive rearrangement steps, where [**3**-DNA]$_I$ and [**3**-DNA]$_{II}$ represent two different intermediate complexes.[30] Although this model describes the kinetic behavior for both DNA sequences, the fit is best for the association of **3** with **oligo B**. In the case of **3** binding to **oligo A**, an additional step was required in order to better account for the fast phase, particularly at lower DNA concentration, which is a key component of the data (Fig. 5b). The additional step fits the kinetic data but the parameters are not sufficiently well constrained to uniquely define the rate constants for the two isomerization steps. Nonetheless, globally fitting data according to both mechanisms yielded well-defined second order association rate constants for the two DNA sequences, which were $3.4 \pm 0.1 \times 10^6 \, M^{-1}s^{-1}$ and $2.5 \pm 0.1 \times 10^6 \, M^{-1}s^{-1}$ (k_1) for **3** binding to **oligo A** and **oligo B**, respectively. Note that an essentially irreversible rearrangement step is consistent with the remarkably slow off-rate of **3** from the target sequence. The rate constants shown in Fig. 5 are listed as irreversible steps only because there was no information to define the reverse rate constants, and should be considered as only net reaction rate estimates. This model can be reconciled with the apparent second order rate constant of $1 \pm 0.5 \times 10^4 \, M^{-1}s^{-1}$ estimated by gel shift assays by allowing the initial binding steps to be readily reversible leading up to the final, largely irreversible step.

Discussion

Previous work with **3** had established the ability to bind the sequence 5′-GATAAGTACTTATC-3′ in a 1:1 stoichiometry with the linkers bound in a minor-major-minor groove topology (Fig. 1c).[15] The DNase I footprinting results presented here have verified that **3** can select and bind this 14 bp sequence in the context of nearly 500 bp (Fig. 2). While no structural verification has been attempted, it is reasonable to assume a similar threading topology on a long piece of DNA in which the linkers alternate in the minor-major-minor groove topology as with the oligonucleotide of the same sequence.[15]

The observed sequence specificity could be the consequence of differences in association rates, dissociation rates or a combination of the two. Based on a 4×10^4-fold slower dissociation rate constant observed for **3** binding its preferred sequence compared to a randomly chosen sequence, it is safe to say that specificity is based largely on differences in the dissociation rates. This conclusion was reinforced by stopped-flow association data that revealed very similar association rate constants for the preferred versus control sequences.

The threading polyintercalation topology is intuitively expected to manifest an extremely slow dissociation due to the seemingly unlikely molecular rearrangements required for full dissociation. In addition, threading intercalator monomers display relatively slow dissociation compared to non-threading monointercalators.[10,11] Indeed, using 100 mM NaCl as benchmark conditions, the observed $k_d = 5.0 \pm 0.5 \times 10^{-7} \, s^{-1}$ for **3** dissociating from its preferred binding sequence corresponds to a dissociation half-life of $t_{1/2} = 16$ days. We envision that multiple individual steps are required for full dissociation, so these values are best thought of as rate-limiting out of several individual rate constants.

The complex topology of a bound threading polyintercalator also raises questions about the detailed mechanism of association. One might predict a rapid and nonspecific association between positively-charged **3** and negatively-charged DNA driven largely by electrostatic attraction. Following that, a large number of distinct states could be envisioned as the molecule samples various topologies and sequences before ending up in the final threading polyintercalation binding mode. The stopped-flow measurements confirmed at least a three-step process, consistent with expectation. Perhaps surprisingly, there were significant similarities between the association rate profiles and rate constants for **3** binding to its preferred versus a random sequence, although detailed curve fitting indicated there may be some subtle mechanistic differences between the two.

Unfortunately, no detailed mechanistic interpretation is possible based on the stopped-flow data alone because spectrophotometric monitoring cannot be used to gain an unambiguous structural understanding of the process. For example, it is possible that the observed decreases in NDI absorbance are the result of a step in which the NDI units are simply buried in a DNA groove and not yet intercalated. In addition, some mechanistic features could be very hard to detect as distinct steps because one could imagine several different bound topologies with just two NDI units intercalated, all of which would have very similar overall absorbance values.

Lack of structural information in the stopped-flow experiment was addressed by using 1D ^{1}H NMR studies and qualitative gel shift measurements. A detailed analysis of the entire spectrum and especially the imino proton signals verified that within the limits of NMR detection (greater than 95%), after the 5.6 min mixing time, **3** was fully bound to its preferred site in the expected threading polyintercalator topology as previously assigned (Fig. 4).[15] Unfortunately, because the complex was fully formed before the first scan was acquired, no detailed rate information could be obtained. On the other hand, the gel shift association experiments were used to provide an estimate of the association rate constant for **3** binding to its preferred sequence in a manner that survives gel electrophoresis. Recall that the gel shift was only observed for **3** binding its preferred sequence, not a randomly chosen sequence, so this measurement is assumed to verify binding to the oligonucleotide in a sequence specific fashion. The approximate association rate constant obtained in this way was $1 \pm 0.5 \times 10^4 \, M^{-1} \, s^{-1}$, which is consistent with full binding under the conditions of the NMR experiment, but is still considerably slower than the slower of the two rate constants determined by stopped-flow. It is likely that the stopped-flow assay reports the initial, and readily reversible association steps, which are followed by a largely irreversible step to form the tight complex observed by NMR and the gel shift assay.

It is interesting to compare the dissociation rate constant measured here to those for other reported long-lived complexes. To the best of our knowledge, a $k_d = 5.0 \pm 0.5 \times 10^{-7} \, s^{-1}$, corresponding to a 16-day half-life, compares favorably to all reported dissociation rate constants for molecules bound to DNA. In fact, we know of only a handful of reported slower dissociation rate constants, including that for the synthetic multivalent *tris*-vancomycin-*tris*-D-Ala complex ($t_{1/2} \approx 200$ days),[31] a few highly engineered antibodies ($t_{1/2} \approx 5 - 26$ days),[32-34] and the avidin-biotin system ($t_{1/2} \approx 89 - 107$ days).[35,36]

Given the lower estimate for the apparent second order rate constant governing the formation of the specific complex of $1 \pm 0.5 \times 10^4$ $M^{-1}s^{-1}$ and the slow dissociation rate, we estimate a net dissocation constant (K_D) of 50 pM for **3** binding its preferred site on **oligo A**. Note that binding to a preferred site on a long piece of DNA will almost certainly be complicated by additional mechanistic steps not seen with the relatively short oligonucleotides used here. We are currently embarking on a comprehensive mechanistic study intended to uncover the rates and individual steps involved when a threading polyintercalator such as **3** "scans" long segments of DNA, locates, and then fully intercalates into a preferred site. The results of these analyses will be reported in due course.

In summary, a roughly 4×10^4-fold preference for a specific 14 bp sequence was determined for **3** relative to a randomly chosen sequence. This specificity was almost entirely due to differences in dissociation rate constants. Importantly, a 16-day half-life at 100 mM NaCl is the slowest we know of for DNA binding molecules, and is presumably sufficiently slow to disrupt biological processes such as transcription, repair, and replication for prolonged periods. Noting that adding between 4 and 5 kcal/mol of binding energy to the dissociation rate constant reaches a bound half-life of 90 years, we will be exploiting the modular nature of our threading polyintercalators to make longer molecules with expected bound half-lives relevant to mammalian lifetimes.

Methods

Tetraintercalator (3) synthesis

The Fmoc-Lys-NDI-(β-Ala)$_3$-Lys-NDI-Gly-resin was prepared as previously described,[14,37] and thereafter following the tetraintercalator synthesis procedure of Lee *et al.*[15] ^{1}H NMR spectra were in agreement with the previously published data,[15] and HRMS-ESI predicted for $C_{128}H_{150}N_{30}O_{34}[M+2H]^{+2}$: 1325.5466, found 1325.8, predicted for $C_{128}H_{151}N_{30}O_{34}[M+3H]^{+3}$: 884.0336, found 884.5, and predicted for $C_{128}H_{152}N_{30}O_{34}[M+4H]^{+4}$: 663.681, found 663.6. Tetraintercalator concentration was determined by the absorbance at 386 nm using an extinction coefficient of 51,300 M^{-1} cm^{-1}.

DNase I footprinting

A double-stranded oligonucleotide containing the tetraintercalator binding site was inserted between the *SfiI* restriction enzyme sites of the pMoPac16 vector.[38] The sequence of the construct was confirmed prior to use. The 467 bp DNA fragment was amplified by PCR using a primer which was 5′-^{32}P end-labeled. The PCR product was purified on a 5% native gel, and the bands were excised and eluted in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH = 7.0). The labeled fragment was incubated for 48 h with **3** at the designated concentration followed by DNase I digestion in 20 mM phosphate buffer (pH = 7.0) and 2 mM MgCl$_2$ for 7 min. A detailed protocol has been described elsewhere.[38] To determine the footprinting sequence, the digestion was compared with an adenine specific sequencing lane.[40]

Gel mobility-shift assays to monitor association and dissociation

The top strand of **oligo A** was labeled with ^{32}P at the 5′ end, annealed to its compliment strand, and gel purified. After excising the band from the gel, the duplex oligonucleotide

was ethanol precipitated and resuspended in ddiH$_2$O. The DNA concentration was quantified with a micro-volume spectrophotometer (Nanodrop ND100). For association experiments, a stoichiometric amount of **3** was added to a solution containing both radiolabeled (50 nM) and unlabeled **oligo A** such that the final DNA concentration ranged from 250 to 2500 nM in 10 mM PIPES buffer (pH = 7.0), 1 mM EDTA, and 100 mM NaCl in a final volume of 12 μL. Aliquots taken at representative times from each DNA concentration were mixed with CT DNA to a final concentration of 250 μM bp to prevent further association. Samples were then mixed with loading buffer (0.25% Bromophenol Blue; 0.25% Xylene Cyanol; 40% sucrose), and loaded on an 8% non-denaturing gel. The gel was dried, visualized digitally, and the amount of bound and unbound radiolabeled **oligo A** was quantified using ImageQuant 4.1 imaging software. For dissociation experiments, radiolabeled **oligo A** (1 μM) was incubated with **3** (1 μM) in 10 mM PIPES buffer (pH = 7.0), 1 mM EDTA, and NaCl concentrations ranging from 25 to 200 mM. Complete association was first verified by gel electrophoresis before conducting the time point dissociation experiments. Once confirmed, a 100-fold excess of non-labeled **oligo A** was added to the **3-oligo A** complex. Aliquots taken at various time points were separated by gel electrophoresis and the bands were quantified as above.

Stopped-flow kinetics

Kinetic data was acquired using a KinTek stopped-flow instrument equipped with 2.5-cm path length optical cell. The absorbance change at 386 nm was monitored for the association and dissociation of **3** with **oligo A** or **oligo B**. Kinetic experiments of DNA association were conducted at constant **3** concentration and varying DNA oligonucleotide concentration. DNA oligos (ranging from 4 to 32 μM) in buffer containing 10 mM PIPES (pH = 7.0), 1 mM EDTA, and 100 mM NaCl were mixed with equal volumes of 4 μM **3** in the same buffer. No absorbance change occurred when **3** was mixed with buffer alone. For the dissociation experiments, pre-incubated **3-oligo B** complexes in 10 mM PIPES (pH=7.0), 1 mM EDTA, and the designated NaCl concentration (50-300 mM) were rapidly mixed with a 4% SDS solution in the same buffer. Data collected at each timescale consisted of one thousand data points. Global data fitting of the averaged kinetic traces was done using the KinTek Explorer software (KinTek Corp, Austin, TX).

1D ^{1}H NMR sample preparation and spectroscopy

Solutions were prepared in 90% H$_2$O / 10% D$_2$O containing 30 mM phosphate buffer (pH = 7.0) and 100 mM NaCl. One solution contained **oligo C** (120 μM), concentration verified by nanodrop spectroscopy (ε = 143,400 L mol^{-1} cm^{-1}, MW = 8,523.6 g mol^{-1}) and the other solution contained **3** (120 μM), as verified by UV-vis spectroscopy (ε = 51,300 M^{-1} cm^{-1}).[11] Control spectra were obtained for **oligo C** at 60 μM final concentration. The kinetic association was monitored by mixing a stoichiometric amount of **3** as prepared above directly into an NMR tube containing **oligo C** as prepared above, to a final 1:1 concentration of 60 μM. Experiments were performed using a Varian DirectDrive 600 MHz spectrometer. 1D NMR spectra in 90% H$_2$O / 10% D$_2$O were taken at 27°C using a jump-return solvent suppression method. All spectra were processed using VNMRJ (Varian, Inc.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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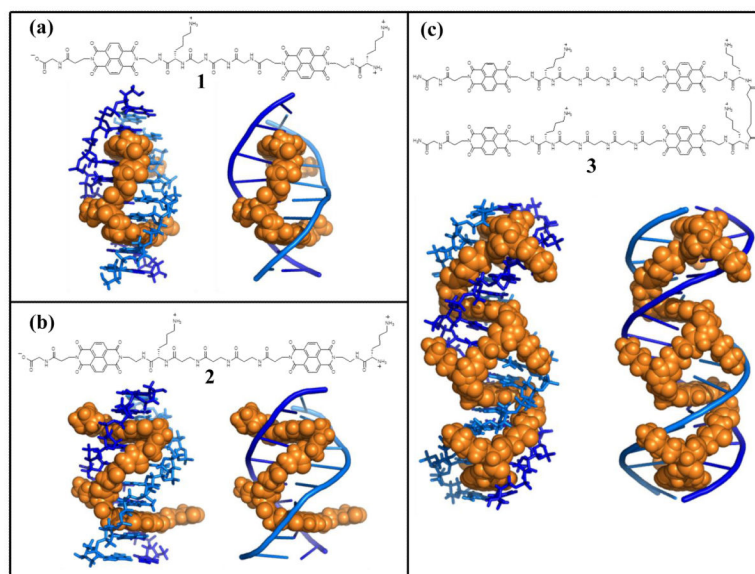
Figure 1. Chemical structures, ligand-DNA complex models, and threading polyintercalation cartoon illustration for NDI polyintercalators

a, Bisintercalator **1** with a view from the major groove of **1**-d(CGGTACCG)$_2$ complex.[13]**b,** Bisintercalator **2** with a view from the minor groove of **2**-d(CGATAAGC)·d(GCTTATCG) complex.[14]**c,** Tetraintercalator **3** with a view of **3**-d(GATAAGTACTTATC)$_2$ complex.[15] Models and cartoon illustrations were produced by The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.
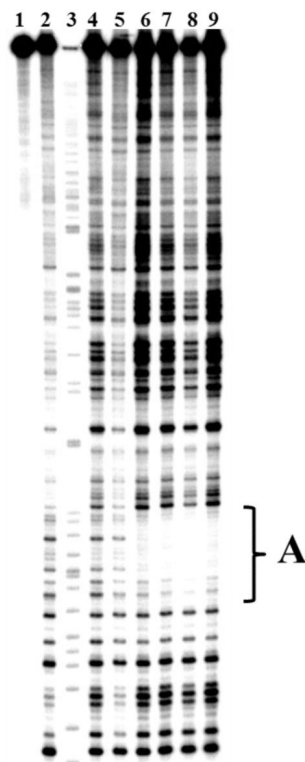
Figure 2. DNase I footprinting of 3 bound to a 467 bp DNA segment
Lane 1 is a control lane without DNase I or **3**. Lanes 2 and 4 contain DNA digested with DNase I for 7 min and 4 min, respectively. Lane 3 is an adenine-specific sequencing lane. In lanes 5 – 9, the DNA fragment was incubated with increasing concentrations of **3** (31, 63, 125, 250, 500 nm), respectively, for 48 hr followed by digestion with DNase I for 7 min. The preferred sequence 5′-GATAAGTACTTATC-3′ (1) is highlighted with a bracket.
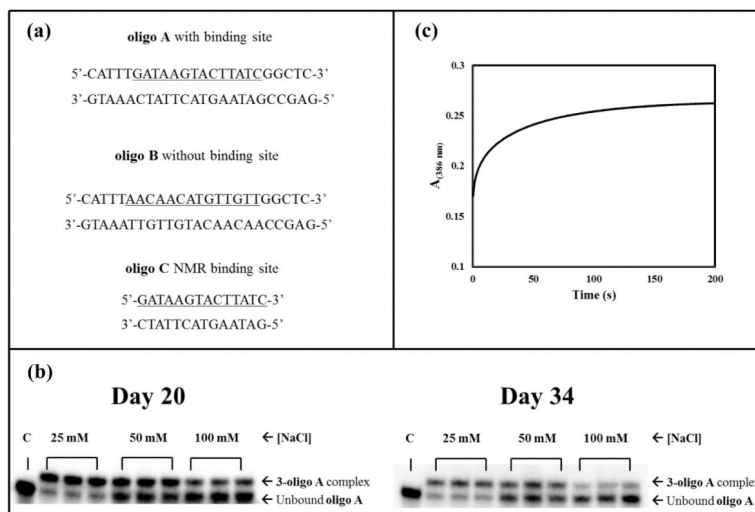
Figure 3. Oligonucleotide duplex sequences used in these studies and representative dissociation gel mobility-shifts from oligo A and stopped-flow dissociation from oligo B

a, Duplex sequences abbreviated as **oligo A-C** as referenced above, with the target sequence underlined for each duplex. **b, 3** dissociating from **oligo A** after 20 days and 34 days of incubation, showing the prolonged dissociation from the preferred binding site. C refers to the control lane which contains **oligo A** in the absence of **3** in 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (pH = 7.0), 1 mM ethylenediaminetetraacetic acid(EDTA), and 100 mM NaCl. All other incubations were run as triplicate measurements in 10 mM PIPES (pH = 7.0), 1 mM EDTA with varying [Na$^+$] as labeled above the respective lanes. Complexed and unbound **oligo A** bands are as labeled. **c,** Representative stopped-flow dissociation trace of **3-oligo B** complex. The equilibrated complex was mixed with equal volumes of 4% sodium dodecyl sulfate (SDS) in 10 mM PIPES (pH = 7.0), 1 mM EDTA, and 100 mM NaCl monitiored at the visible absorbance maximum of 386 nm, showing the rapid dissociation from a control sequence. The data was fit using a double-exponential equation.
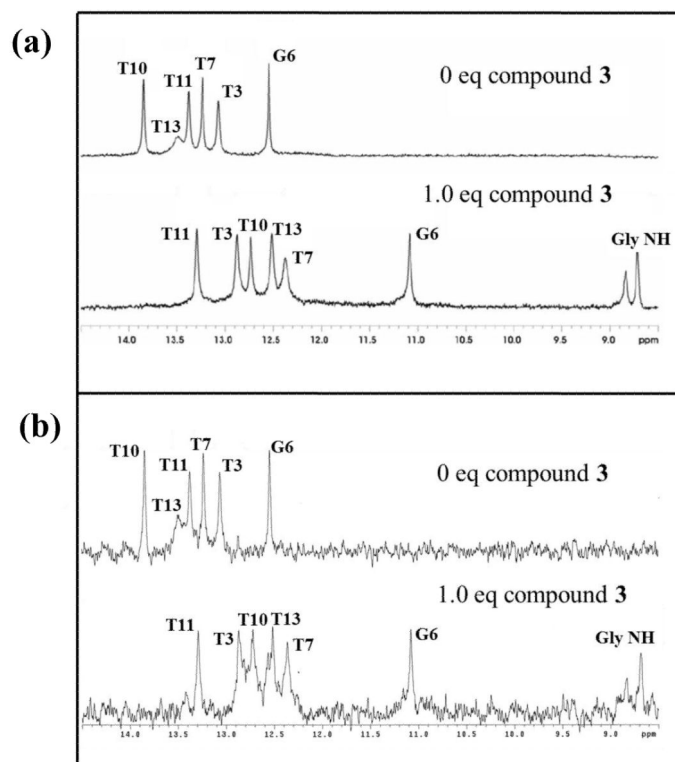
Figure 4. 1D ^{1}H NMR kinetic titrations of 3 binding to oligo C
All of the NMR peaks are labeled corresponding to the single letter designation for the DNA nucleobase as it would be numbered from 5′ to 3′ for **oligo C**. **a,** 1D ^{1}H NMR spectra of **3** titration into d(GATAAGTACTTATC)$_2$ completed by Lee *et. al*[15] with the spectra shown for 0 and 1.0 equivalents of **3** added, respectively, showing the emergence of a new set of distinct imino proton peaks. **b,** 1D ^{1}H NMR spectra of **oligo C** with both 0 and 1.0 equivalents of **3** added, respectively. The 1:1 association kinetic run with 1.0 eq of **3** was produced after 5.6 min at a final concentration of 60 μM **3**:**oligo C** in 30 mM phosphate buffer (pH = 7.0), with the distinct complexed imino proton peaks indicating structural verification for complete association of the **3-oligo C** complex.
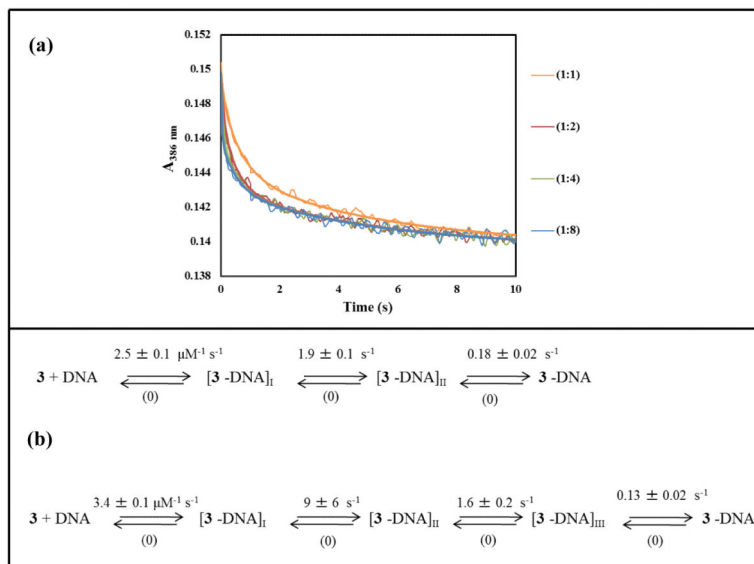
Figure 5. Stopped-flow association traces of 3 from oligo B with proposed models for association of 3 to both oligo A and oligo B

a, Representative stopped-flow absorbance traces of the association of **3** (2 μM) with **oligo B** at four different DNA concentrations (2, 4, 8, 16 μM) in 10 mM PIPES buffer (pH = 7.0), 1 mM EDTA, 100 mM NaCl. The solid lines represent the best global fit to a three-step consecutive mechanism using KinTek Explorer Software. A similar absorbance trace was obtained for the association of **3** to **oligo A** (Fig. S5, Supplementary Information). **b,** Rate constants were derived from global fitting the concentration dependence of the data series to a three step model. Both models assume irreversible steps toward complex formation. The data for the association of **3** with **oligo B** best fit the top model, while the data for the association of **3** with **oligo A** best fit the bottom model.

Table 1

Cumulative data for the dissociation of **3** from both **oligo A** and **oligo B** at varying [Na$^+$]. Dissociation rate constants of **3** from **oligo A** calculated from the monoexponential fits to the gel shifts. Error levels estimated at ± 17%. For **oligo B**, A$_1$ and A$_2$ are the relative amplitudes for the double exponential according to $k_{d,app} = A_1 k_1 + A_2 k_2$. The estimates of the rate constants fit a double exponential equation with a standard error less than 1%. In order to obtain the faster rates and corresponding amplitudes, the fits to the traces obtained at 10 s intervals were coerced to the slower rates and their amplitudes obtained from the 200 s traces.

	Oligo A		Oligo B				
[Na$^+$] (mM)	**$k_d \times 10^{-7}$ (s^{-1})**	**$t_{1/2}$ (days)**	**k_1 (s^{-1})**	**A$_1$ (%)**	**k_2 (s^{-1})**	**A$_2$ (%)**	**k_d (s^{-1})**
25	2.3	32	-	-	-	-	-
50	3.3	23	0.27	30	0.012	70	0.088
100	5.0	16	0.29	34	0.019	66	0.11
150	5.7	13	0.38	35	0.031	65	0.15
200	9.3	8	0.39	40	0.038	60	0.18
300	-	-	0.48	39	0.055	61	0.22