Supporting Information for

Synthetic DNA binders for fluorescence sensing of thymine glycol-containing DNA duplexes and inhibition of endonuclease activity

Yusuke Sato,[a]* Yoshihide Takaku,[a] Toshiaki Nakano,[b] Ken Akamatsu,[b*] Dai Inamura,[a] and Seiichi Nishizawa[a]*

[a] *Department of Chemistry, Graduate School of Science, Tohoku University, Aoba-ku, Sendai, 980-8578, Japan.*
[b] *DNA damage chemistry research group, Institute for Quantum Life Science, National Institutes for Quantum Science and Technology, Kizugawa, 619-0215, Japan.*

Experimental

Reagents: All of the DNAs were custom-synthesized and HPLC-purified by Nihon Gene Reserach Laboratoriers Inc. (Sendai, Japan) or Tsukuba Oligo Service Co. Ltd. (Ibaraki, Japan). *Escherichia coli* Endo III was purchased from New England Biolab (Ipswich, MA, USA). Other reagents were commercially available analytical grade and were used without further purification. MALDI-TOF-MS spectra were recorded on a Bruker Daltonics autflex Speed-S1 (Bruker, Germany). The concentrations of DNAs were determined according to the literature.[1] Water was deionized (≥18.0 MΩ cm specific resistance) by an Elix 5 UV water purification system and a Milli-Q Synthesis A10 system (Millipore Corp., Bedford, MA, USA), followed by filtration through a BioPak filter (Millipore Corp.).

Unless otherwise mentioned, all measurements were performed at 20°C in PBS buffer (pH 7.4). Before measurements, the sample solutions were annealed in order to assure the formation of DNA duplexes. Annealing conditions were as follows: heated at 75°C for 10 min, and gradually cooled to 5°C (3°C/min), after which the solution temperature was raised again to 20°C (1°C/min).

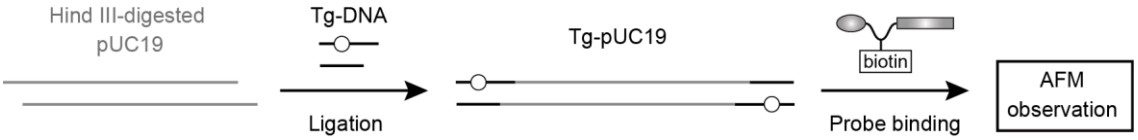
UV-visible spectra and fluorescence spectra measurements: Absorption and fluorescence spectra were measured at 20°C with a JASCO model V-570 UV–vis spectrophotometer and FP-6500 spectrofluorophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan), respectively. Both instruments were equipped with thermoelectrically temperature-controlled cell holders. Measurements of absorption and fluorescence spectra were done using a 1.0 × 10 mm quartz cell (optical path length: 10 mm) and a 3 × 3 mm quartz cell, respectively. Fluorescence quantum yield (Φ) of the probe was determined relative to fluorescein (Φ = 0.93).[2] The dissociation constant (K_d) values for DNA binding were obtained by the fluorescence titration experiments as previously described.[3]

Melting temperature measurements: Absorbance of DNAs was measured at 260 nm as a function of temperature using an UV-vis spectrophotometer Model UV-2450 (Shimadzu Corp., Kyoto, Japan) equipped with a thermoelectrically temperature-controlled micro-multicell holder (8 cells; optical path length = 1 mm). The

temperature ranged from 10°C to 100°C with a heating rate of 0.5°C/min. The resulting absorbance versus temperature curve was analyzed by a differential method to determine T_m values.

Evaluation of inhibitory activity by gel electrophoresis: 65 nM Endo III was incubated with 1.0 μM Tg-DNAs in the absence and presence of DML-Dab-TO (0-100μM), at 37°C for 30 min in a reaction buffer (20 mM Tris-HCl, 1.0 mM ETDA and 1.0 mM DTT, pH 8.0). After mixing with loading buffer (Wako, Japan), samples were loaded onto a 16% polyacrylamide gel containing 8.0 M urea, 89 mM Tris borate (pH 8.3) and 2.0 mM EDTA. Electrophoresis was conducted for 1 h at 10 mV. After electrophoresis, the gels were stained by SYBR green II, visualized and analyzed by LAS-4000 (Fuji Film, Tokyo). Half maximum inhibitory concentration (IC_{50}) was determined according to the literature.[4]

AFM imaging of DNA containing Tg based on the interaction between biotin-carrying DML-Dab-TO and streptavidin: A model DNA containing Tg was prepared to confirm the recognition of Tg by DML-Dab-TO. A long DNA with more than ~1 kbp is needed for AFM imaging to distinguish a DNA molecule from other impurities. Briefly, Tg-containing double-stranded (ds) DNA oligomer with a Hind III sticky end was ligated to Hind III-digested pUC19 plasmid DNA. The scheme for AFM experiments was shown in Scheme S1.



Scheme S1. Schematic illustration for preparation of DNA samples and AFM experiments.

1. Preparation of Hind III-digested pUC19 (pUC19/Hind III)

   One hundred microliters of pUC19 (2686 bp, 1.0 μg/μL in TE, Bayou Biolabs, Metairie, LA, USA) was mixed with 10 μL of 10 x buffer (NE Buffer2, New England Biolabs (NEB), Ipswich, MA, USA) and 1.0 μL of BSA (10 mg/mL), followed by incubation with 5.0 μL of Hind III-HF (NEB) overnight at 37°C. The solution was purified using two spin columns (NucleoSpin gDNA Clean-Up, Macherey-Nagel, Düren, Germany) to obtain pUC19/Hind III solution (~ 0.6 μg/μL in TE, ~100 μL). The digestion was confirmed by gel electrophoresis (1% agarose/0.5 x TBE, 3.7 V/cm, 4h).
2. Preparation of Tg-containing ds DNA oligomer with a Hind III sticky end

   Twenty microliters of a Tg-containing DNA oligomer, pAGCTTACGCCCGCGATgACGCCCGCGA (0.1 mM/TE, Tsukuba Oligo Service) and 20.5 μL of its complementary sequence, TCGCGGGCGTATCGCGGGCGTA (0.1 mM/TE), was mixed and incubated at 95°C for 3 min, followed by cooling naturally to room temperature to form Tg-containing dsDNA oligomer (~50 μM/TE). The product was confirmed by gel electrophoresis (4% MetaPhor Agarose (LONZA, Basel, Switzerland)/0.5 x TBE, 3.7 V/cm, 4h).

3. Ligation of Tg-containing dsDNA oligomer with pUC19/Hind III

   Six microliters of pUC19/Hind III (~2 pmol) and 4.0 μL of Tg-containing dsDNA oligomer (~200 pmol) were mixed with 40 μL of TE and 100 μL of a solution for ligation (Ligation High, Takara Bio, Shiga, Japan) on ice, followed by incubation at 16°C for 1h. The reaction solution was treated by a spin column (NucleoSpin gDNA Clean-Up) (~100 μL/TE). Furthermore, the obtained solution was purified by extraction from an agarose gel after electrophoresis (1% agarose/0.5xTBE) using an extraction kit (Zymoclean Gel Recovery Kit, Zymo Research, Irvine, CA, USA). Concentration of the purified Tg-pUC19 (Scheme S1) was ca.100 ng/μL/TE. The ligation reaction was confirmed by reproduction of the dsDNA oligomer from Tg-pUC19 by Hind III treatment.

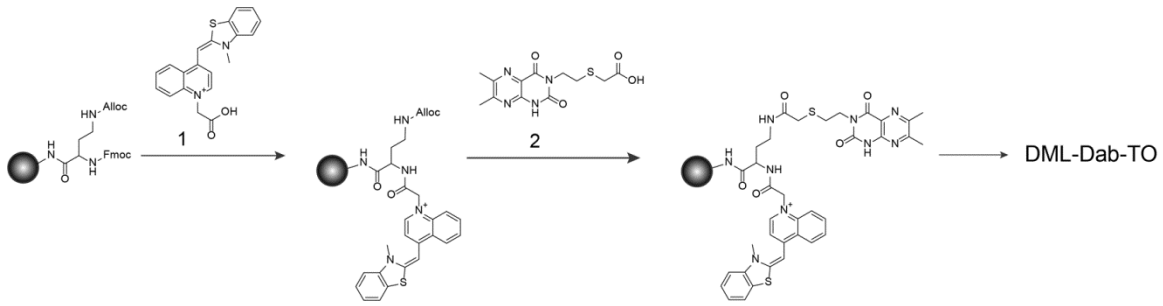
4. AFM observation of a Tg site in Tg-pUC19 using biotin-carrying DML-Dab-TO and streptavidin

   Conjugation of biotin-carrying DML-Dab-TO to Tg-pUC19 was prepared with reference to the previous report.[5] In brief, Tg-pUC19 was incubated with biotin-carrying DML-Dab-TO in TE at 37°C for 2h. Free biotin-carrying DML-Dab-TO was removed by a Centri-Sep spin column using PBS as an elution buffer. The obtained biotin-carrying DML-Dab-TO-labeled Tg-pUC19 was incubated with streptavidin in PBS at 37°C for 3h. Free streptavidin was removed by a Chroma spin TE200 column using TE.

   One microliter of the streptavidin-labeled DNA was mixed with 1.0 μL of 40 mM $NiCl_2$. The sample was adsorbed onto a freshly cleaved mica plate for 5 min at room temperature, and the surface was gently washed twice with 10 μl of MilliQ water. AFM images were acquired using a high speed-scanning AFM system (Nano Live Vision, RIBM, Japan) equipped with an ultra-short cantilever (Nano World USC-F1.2-k0.15). Scanning was performed using the tapping mode in MilliQ water. All images were recorded with an image acquisition speed of 1 frame/s.

Synthesis of DML-Dab-TO: DML-Dab-TO was synthesized using Biotage Intiator+ microwave peptide synthesizer (Biotage, Uppsala, Sweden). Fmoc-Dab(Alloc)-OH was first loaded onto a Rink-Amide-Chem Matrix resin (Biotage), followed by the deprotection of Fmoc group by piperidine. TO unit having a carboxylated group (**1**)[6] was coupled to the main chain of Dab residue on the resin. The alloc group was then deprotected by treating with tetrakis(triphenylphosphine) palladium (0) and dimethylamine borane in dichloromethane, followed by coupling with carboxylate-terminated ethyl spacer-containing DML derivative (**2**).[7] Cleavage from the resin was performed using a mixture of trifluoroacetic acid (TFA)/m-cresol (95/5, v/v). After the filtration, the probes were then precipitated in cold diethyl ether. The crude products were purified using a reverse-phase HPLC system (pump, PU-2086 Plus ×2; mixer, MX 2080-32; column oven, CO-1565 (55°C); detector, UV-2070 plus and UV-1570M (JASCO) equipped with a C18 column (Inertsil ODS3; GL Sciences Inc., Tokyo, Japan) using a gradient of water/acetonitrile containing 0.2% TFA. DML-TO was verified by MALDI-TOF-MS and ^{1}H-NMR.
^{1}H-NMR (500 MHz, DMSO-d_6): δ = 12.04 (s, 1H), 8.84 (d, J = 7.9 Hz, 1H), 8.78 (d, J = 8.2 Hz, 1H), 8.51 (d, J = 7.3 Hz, 1H), 8.05 (dd, J = 15.1, 6.6 Hz, 2H), 7.96 (t, J = 7.6 Hz, 1H), 7.84 (t, J = 9.5 Hz, 2H), 7.73 (t, J = 7.5 Hz, 1H), 7.64 (t, J = 7.8 Hz, 1H), 7.55 (s, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.40 (d, J = 7.3 Hz, 1H), 7.19 (s, 1H), 6.98 (d,

J = 7.0 Hz, 1H), 5.37 (d, *J* = 5.2 Hz, 2H), 4.07 (t, *J* = 7.9 Hz, 5H), 3.18 (s, 2H), 2.79 (t, *J* = 7.2 Hz, 2H). MALDI-TOF MS for DML-Dab-TO [M+]; calcd 740.24, found 740.90.



Scheme S2. Synthesis scheme of DML-Dab-TO.

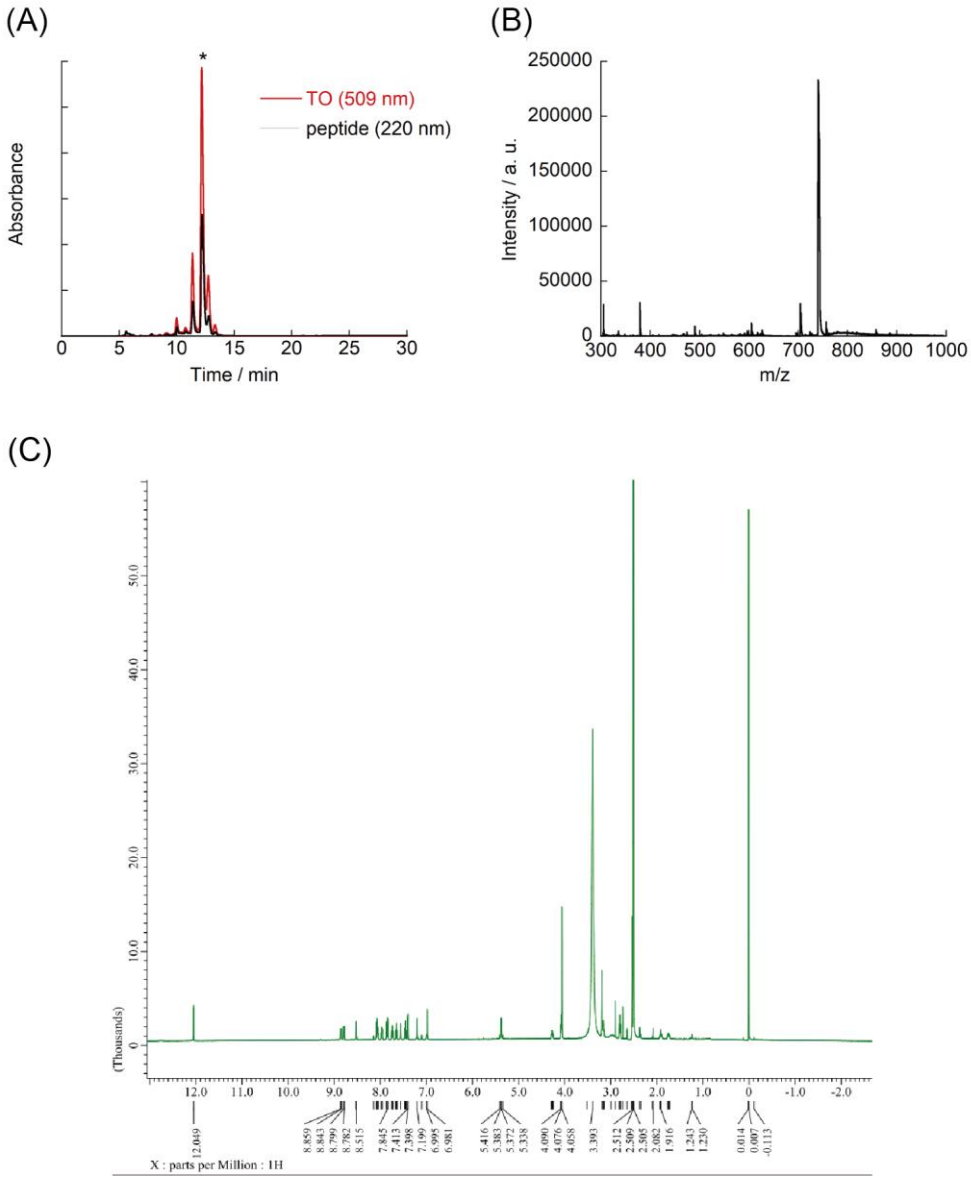


Figure S1. (A) HPLC profile for the purification of DML-Dab-TO. Gradient condition: 36-40 % CH_3CN (0.2 % TFA) in H_2O (0.2 % TFA) during 30 min. The peak (*) was collected and identified as the purified probe. (B) MALDI-TOF-MS spectra of the purified DML-Dab-TO. (C) ^1H NMR of the purified DML-Dab-TO in DMSO-d_6.

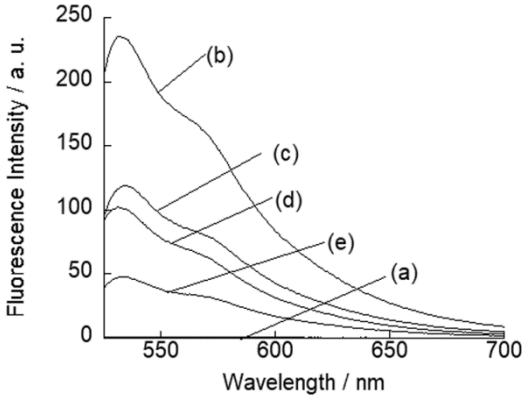


Figure S2. Fluorescence spectra of DML-Dab-TO (500 nM) in the (a) absence and presence of DNAs (500 nM) carrying (b) A, (c) C, (d) T or (e) G nucleobase opposite Tg. Excitation, 512 nm. Other conditions were the same as those given in Figure 2 in the main text.

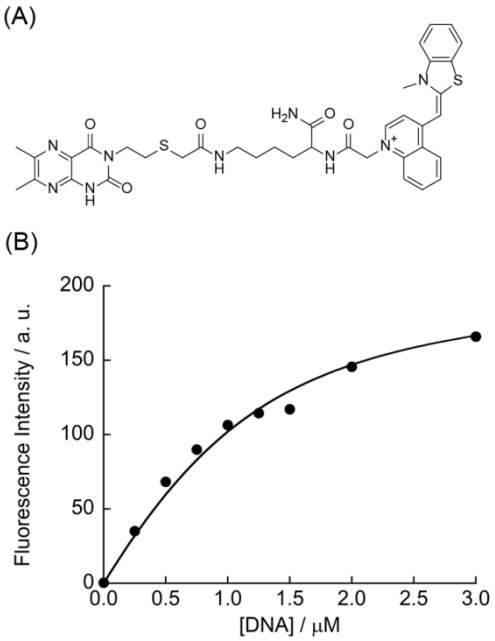


Figure S3. Examination of the effect of the spacer length between DML and TO units on the binding affinity for Tg-DNA (ATgA). (A) Chemical structure of the conjugate (named DML-Lys-TO[7]) with the longer spacer relative to DML-Dab-TO (cf. Figure 1 in the main text). (B) Fluorescence titration curve of DML-Lys-TO (1.0 μM) for the binding to Tg-DNA (0-3.0 μM). Excitation, 512 nm. Analysis, 533 nm. Other conditions were the same as those given in Figure 2 in the main text.

As shown in Figure S3B, DML-Lys-TO with the long spacer exhibited the light-up response upon binding to Tg-DNA, as observed for DML-Dab-TO (cf. Figure 2 in the main text). However, its binding affinity was found to be smaller (K_d = 480 ± 160 nM) compared to DML-Dab-TO. This indicates the longer spacer is unfavorable for the binding to Tg-DNAs.

Table S1. Summary of the dissociation constants (K_d) and the fluorescence quantum yield of DML-Dab-TO in the bound state with Tg-DNAs

Sequence: 5'-ACG CCC GCG NTgN CGC CCG CGA-3'/ 3'-TGC GGG CGC N'AN' GCG GGC GCT-5'	K_d / nM	Φ_{bound} [a]
ATgA (N = A, N' = T)	130 ± 21	0.32
GTgG (N = G, N' = C)	42 ± 9.9	0.32
CTgC (N = C, N' = G)	11 ± 3.3	0.55
TTgT (N = T, N' = A)	160 ± 30	0.30

[a] [DML-Dab-TO] = 1.0 μM, [Tg-DNA] = 1.0 μM.

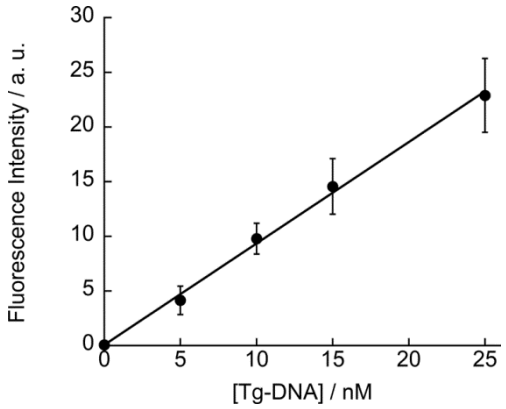


Figure S4. The linear fitting analysis for the determination of the limit of detection (LOD). [DML-Dab-TO] = 1.0 μM, [Target Tg-DNA (CTgC)] = 0-25 nM. Other measurement conditions are the same as those given in Figure 2 in the main text. Excitation, 512 nm. Analysis 533 nm. Temperature, 20°C.

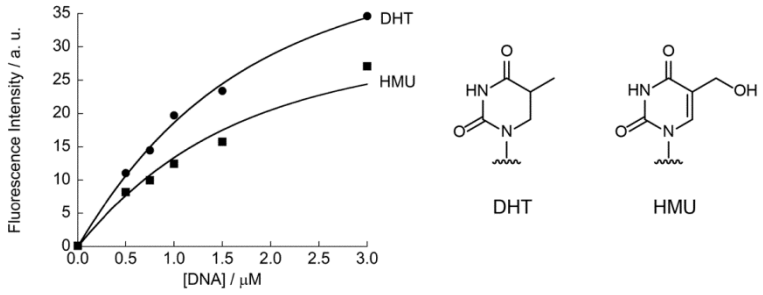


Figure S5. Fluorescence titration curves for the binding of DML-Dab-TO (1.0 μM) to HMU or DHT-carrying DNA duplexes (0-3.0 μM; 5'-ACG CCC GCG AZA CGC CCG CGA-3'/3'-TGC GGG CGC TAT GCG GGC GCT-5', Z = HMU or DHT). Chemical structures of DHT and HMU were also shown. Other solution conditions were the same as those given in Figure 2 in the main text. Temperature, 20°C. Excitation, 512 nm. Analysis, 533 nm.

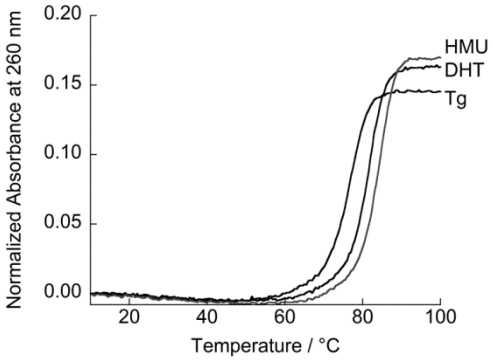


Figure S6. Thermal denaturation profiles of Tg, HMU, or DHT-containing DNA duplexes (1.0 μM; 5'-ACG CCC GCG AZA CGC CCG CGA-3'/3'-TGC GGG CGC TAT GCG GGC GCT-5', Z = Tg, HMU or DHT) in PBS buffer (pH 7.4).

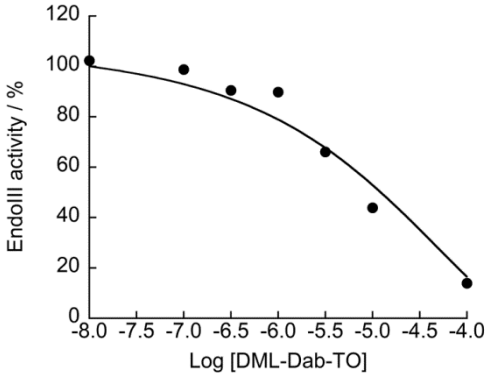


Figure S7. Evaluation of inhibitory activity of DML-Dab-TO for Endo III using ATgA sequence (cf. Table S2).

IC_{50} value was estimated as > 20 μM, which indicates inferior inhibition of DML-Dab-TO for Endo III when ATgA sequence was used as a subtrate.

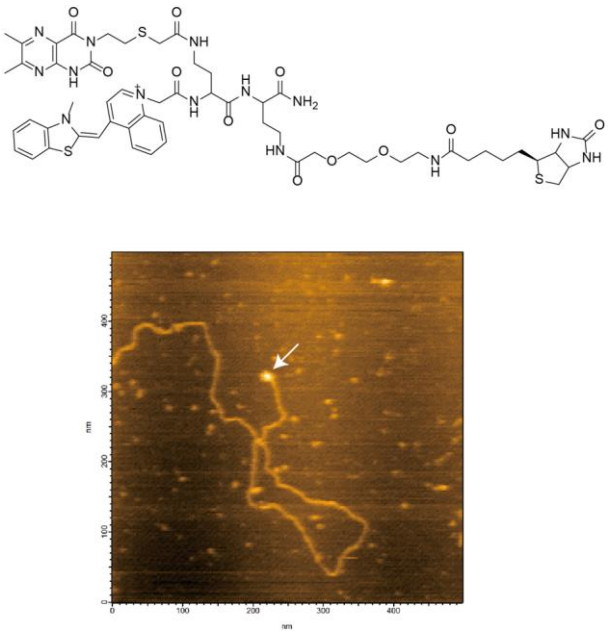


Figure S8. AFM imaging of DNA containing Tg. White arrow shows the streptavidin tag with Tg-DNAs in combination with the biotin unit-carrying DML-Dab-TO conjugate.

References

1. Puglisi, J. D.; Tinocco, I. *Methods Enzymol.* **1989**, *180*, 304-325.
2. Sjoback, R.; Nygren, J.; Kubista, M. *Spectrochim. Acta, Part. A* **1995**, *51*, L7–L21.
3. Sato, Y.; Kudo, M.; Toriyabe, Y.; Kuchitsu, S.; Wang, C.-X.; Nishizawa, S.; Teramae, N. *Chem. Commun.* **2014**, *50*, 515–517.
4. Jun, W. W.; Wilson, D. L.; Kietrys, A. M.; Lotsof, E. R.; Conlon, S. G.; David, S. S.; Kool, E. T., *Angew. Chem. Int. Ed.* **2020**, *59*, 7450-7455.
5. Xu, X.; Nakano, T.; Tsuda, M.; Kanamoto, R.; Hirayama, R., Uzawa, A.; Ide, H. *Nucleic Acids Res.* **2020,** *48*, e18.
6. Sato, T.; Sato, Y.; Nishizawa, S., *J. Am. Chem. Soc.* **2016**, *138*, 9397-9400.
7. Sato, Y.; Saito, H.; Aoki, D.; Teramae, N.; Nishizawa, S. *Chem. Commun.* **2016**, 52, 14446-14449.