Supplementary Information

Fluorescent nucleobase analogue for cellular visualisation and regulation of immunostimulatory CpG oligodeoxynucleotides

Materials and Methods

1. Solid-phase Synthesis of Oligodeoxynucleotides

Oligodeoxynucleotides (ODNs) were synthesised on solid supports using ^{Thex}**T** phosphoramidites (compound **1**, Figure S1) or commercially available standard DNA/RNA phosphoramidites (Glen Research and Sigma-Aldrich). Compound **1** was prepared according to a previously described procedure.[1] Other reagents for solid-phase synthesis were purchased from FUJIFILM Wako Pure Chemicals (Wako), Sigma-Aldrich, Glen Research, and ChemGenes. The solvents were purchased from Wako and Nacalai Tesque. Solid-phase synthesis was performed at a 1 μmol scale on an M-2-MX DNA/RNA synthesiser (Nihon Techno Service) according to a published procedure.[1] The solid support was treated with 1.0 mL of a 50:50 ratio of methylamine ($MeNH_2$) (40 wt. % in water) and ammonia (NH_3) (28 wt. % in water) at room temperature for 15 min and then at 65 °C for 15 min to cleave and deprotect the oligonucleotides. The synthesised ODNs were purified using Glen-Pak DNA purification cartridges (Glen Research). Following elution, the samples were lyophilised, and the resulting residue was dissolved in distilled water (Otsuka Pharmaceutical). The products were characterised by electrospray ionisation-mass spectrometry (ESI-MS) using a Thermo Exactive Plus spectrometer (Thermo Fischer Scientific) (Table S1). The other ODNs were purchased from Integrated DNA Technology. The concentrations of ODNs were determined by measuring the absorbance at 260 nm using a NanoDrop ND-2000 spectrophotometer (Thermo Fischer Scientific).

Table S1. Electrospray ionisation-mass spectrometry (ESI-MS) data of synthesized ODNs[a]

Name	Sequence (X = ^{Thex}T)	Ion	MS	
			Calcd.	Found
ODN^{Thex}T1	**X**CCATGACGTTCCTGATGCT	$[M-8H]^{8-}$	820.575	820.575
ODN^{Thex}T5	TCCA**X**GACGTTCCTGATGCT	$[M-8H]^{8-}$	820.575	820.578
ODN^{Thex}T10	TCCATGACG**X**TCCTGATGCT	$[M-8H]^{8-}$	820.575	820.575
ODN^{Thex}T11	TCCATGACGT**X**CCTGATGCT	$[M-8H]^{8-}$	820.575	820.574
ODN^{Thex}T14	TCCATGACGTTCC**X**GATGCT	$[M-8H]^{8-}$	820.575	820.577
ODN^{Thex}T17	TCCATGACGTTCCTGA**X**GCT	$[M-8H]^{8-}$	820.575	820.580

[a]All phosphate linkages were replaced with phosphorothioates.

2. UV-Visible Absorbance and Emission Spectra of Oligodeoxynucleotides

The UV-visible absorbance spectra were measured from 220 to 500 nm at room temperature using a UV-1800 spectrophotometer (Shimadzu) with a 1 cm path length cuvette. Fluorescence emission spectra were recorded from 410 to 700 nm at room temperature on a Fluoromax-4 (Horiba) with both excitation and emission slits set at 5 nm. The excitation wavelength was 400 nm. All measurements were recorded using 2 μM sample solutions in distilled water.

3. Cell Culturing

For cytokine release assay, macrophage-like murine cells RAW 264.7 (ATCC) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Shimadzu Diagnostics) supplemented with 10% heat-inactivated foetal bovine serum (HI-FBS, Thermo Fisher Scientific), 0.2% sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Nacalai Tesque) at 37 °C in humidified air containing 5% CO_2. For imaging studies, RAW 264.7 cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium High Glucose (DMEM, Wako) supplemented with 10% HI-FBS (Biowest) and 1% Penicillin-

Streptomycin-L Glutamine Solution (X100) (Wako) at 37 °C in humidified air containing 5% CO2.

4. Confocal Microscopic Observation

RAW 264.7 cells (ATCC) were seeded on μ-Slide 8 well dishes (ibidi) at a density of 1.5×10^5 cells/well and cultured for 1 d prior to treatment. The medium was replaced with Opti-MEM containing 10 μM ODNs and incubated for 4 hours at 37 °C in humidified air containing 5% CO_2. After treatment with ODNs, the cell samples were washed three times with phosphate-buffered saline (PBS, Wako), and the medium was replaced with Dulbecco's modified Eagle's medium with HEPES (DMEM-HEPES, Wako) supplemented with 2% HI-FBS (Biowest), which was used as the medium for the imaging studies. For nuclear co-staining, the cells were treated with 10 μM ODNs following standard protocols. After washing, the samples were incubated in imaging medium containing 20 μM of DRAQ5 (Biostatus) at 37 °C for 30 minutes. Cells treated with DRAQ5 were analysed without further washing. Live-cell images were acquired using a confocal microscope FV-3000 (Olympus) with excitation at 405 nm and emission detection between 420 nm and 500 nm for ^{Thex}**T**-modified ODNs, with excitation at 640 nm and detection of emission between 650 nm and 740 nm for DRAQ5.

5. Cytokine Release from RAW 264.7 Cells

RAW 264.7 cells were seeded in a 96-well plate at a density of 5.0×10^4 cells/well and incubated for 1 d before treatment. The medium was then replaced with Opti-MEM containing 400 nM ODNs (four wells per DNA). After incubation for 8 h at 37 °C in humidified air containing 5% CO_2, the 96-well plates were centrifuged at 300 g for 3 min, and then collected supernatants were stored at −70 °C until use. The concentrations of tumour necrosis factor (TNF)-α in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using mouse TNF-α ELISA kits (BD Biosciences).

6. Molecular modelling studies

Molecular modelling was performed using the Molecular Operating Environment (MOE) software package. The binding models of DNA containing ^{Thex}**T** and TLR9 complexes were constructed based on the previously reported agonistic ODN 1668-bound TLR9 structure, 3WPC.[2] The Amber force field was applied to all molecules to represent intermolecular interactions of agonistic ODN within TLR9 structures, and energy optimisation was performed until an RMS gradient of less than 0.001 kcal mol^{-1} Å was achieved. For energy minimisation water molecules were added to produce a distance of 10 A from the solute to droplet sphere boundaries and sodium counter ions were added to neutralise the system.

7. Statistical Analysis

Differences were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Tukey−Kramer tests for multiple comparisons.

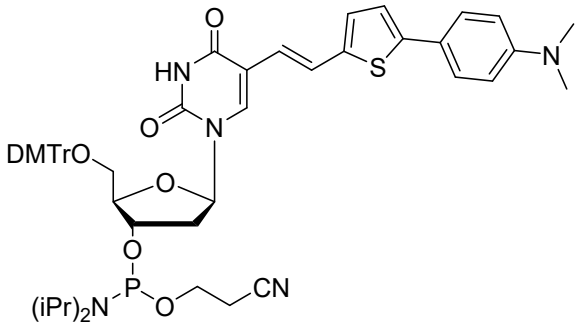

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Figure S1. Chemical structure of ^{Thex}**T** phosphoramidite **1**.

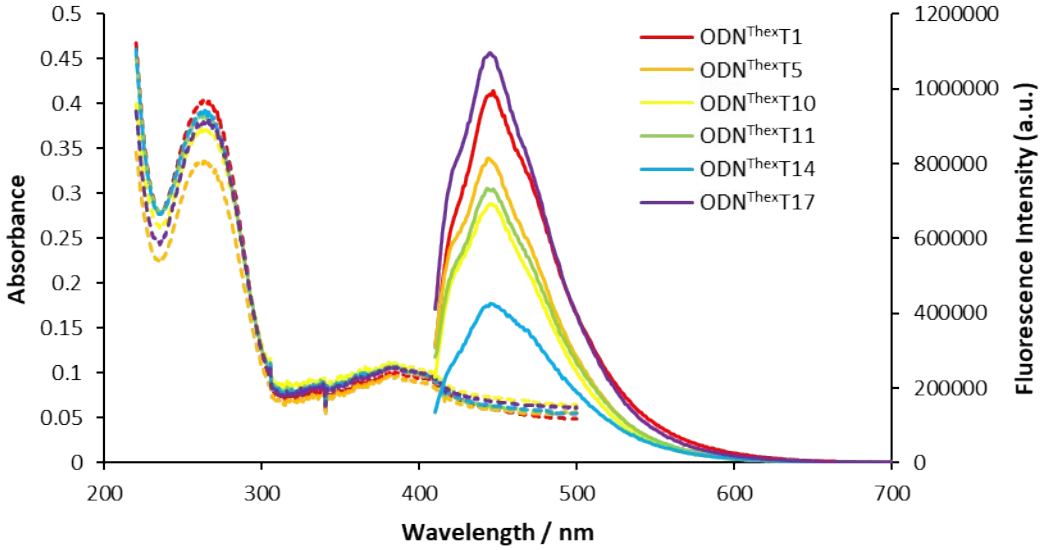

Figure S2. Absorption (dashed line) and fluorescence (solid line) spectra of ^{Thex}**T**-modified ODNs (2 μM) in distilled water.

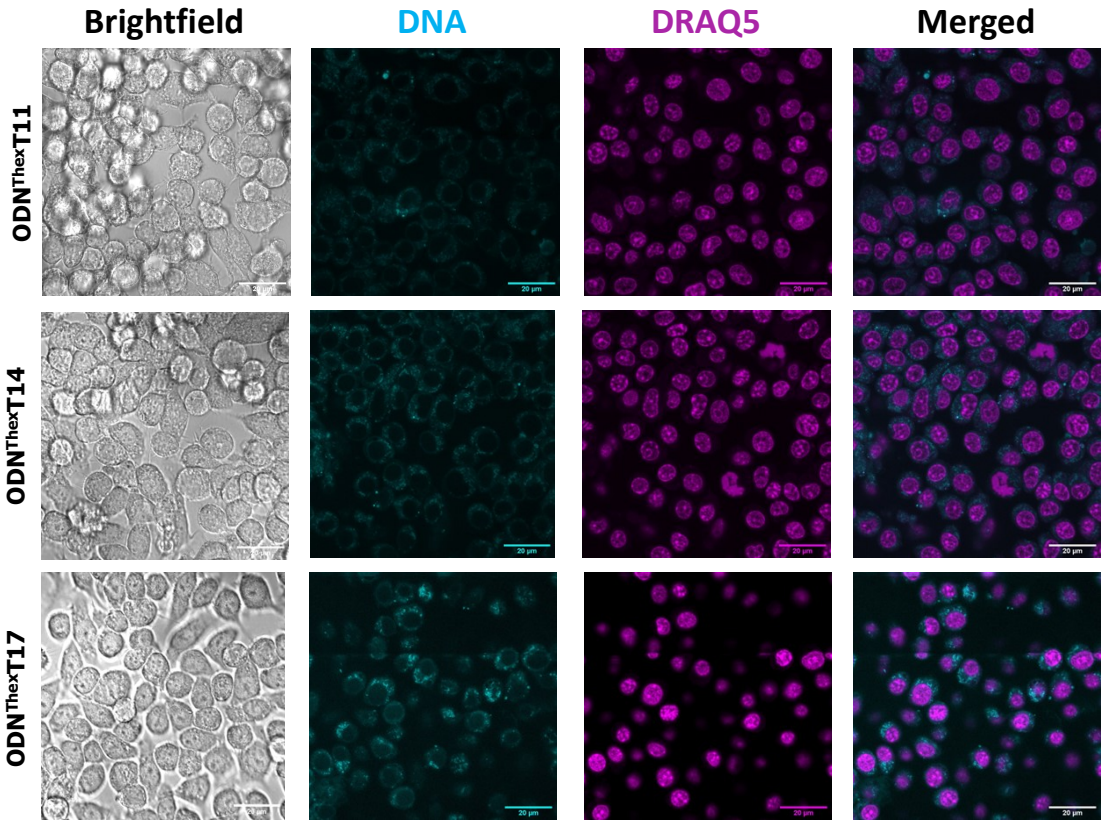

Figure S3. Confocal microscopic images of RAW 264.7 cells co-stained with ^{Thex}**T**-modified DNA (10 μM) for 4 hours and DRAQ5 (20 μM) for 30 min. Nuclei were stained with DRAQ5. Bright-field images are included to provide additional cellular context.

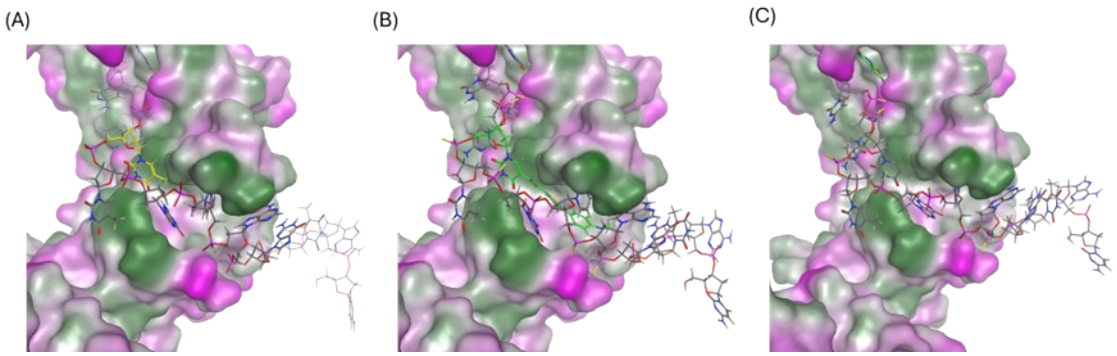

Figure S4. Molecular surface models of TLR9–agonistic DNA binding regions; TLR9 with (A) unmodified ODN 1668_12 mer (B) **ODN^{Thex}T11**_12 mer (C) **ODN^{Thex}T14**_12 mer based on PDB 3WPC. Surface colours represent the hydrophilic (pink), neutral (grey), and lipophilic (green) regions. The DNA molecules are shown in stick representation.

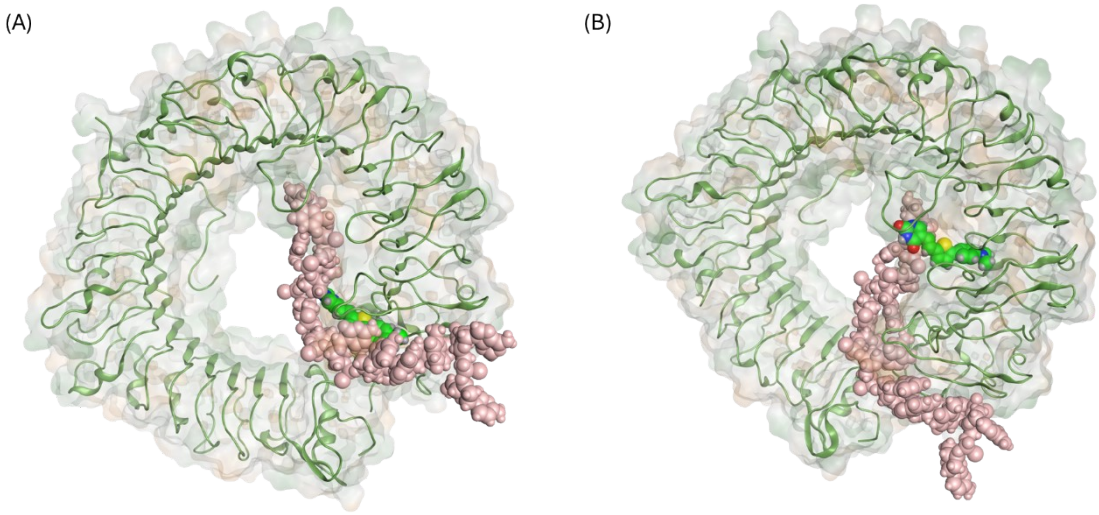

Figure S5. Monomer structural models of TLR9–agonistic DNA complexes; (A) Plausible energy minimized binding model of TLR9 with **ODN^{Thex}T11**_12 mer (B) **ODN^{Thex}T14**_12 mer based on PDB 3WPC. The DNA molecules are shown in a space-filling representation and are depicted in green.

References

1. T. Kumagai, B. Kinoshita, S. Hirashima, H. Sugiyama, S. Park. *ACS Sens.* 2023, **8**, 923–932.

2. U. Ohto, T. Shibata, H. Tanji, H. Ishida, E. Krayukhina, S. Uchiyama, K. Miyake, T. Shimizu. *Nature* **2015**, *520*, 702–705.