Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information for

Design of a fluorogenic PNA probe capable of simultaneous recognition of 3'-overhang and double-stranded sequences of small interfering RNAs

Takaaki Tanabe, Takaya Sato, Yusuke Sato* and Seiichi Nishizawa*

Department of Chemistry, Graduate School of Science, Tohoku University, Japan, Sendai 980-8578, Japan.

Experimental

Reagents

NovaSyn TGR Resin was purchased from Merck Millipore (Billerica, MA). Fmoc-protected amino acids were purchased from AAPPTec (Louisville, KY). Fmoc/Bhoc protected PNA monomers were purchased from Panagene (Hannover, Germany). RNAs were custom synthesized and HPLC purified (>90-97%) by Sigma-Genosys (Hokkaido, Japan). The concentration of the RNAs was determined from the absorbance at 260 nm at 85°C using the molar extinction coefficient provided by the manufacturer. Other reagents were commercially available analytical grade and were used without further purification. Water was deionized (> 18.2 MΩ cm specific resistance) with an Elix 5 UV water purification system and a Milli-Q Synthesis A10 system (Millipore Co., Bedford, MA), followed by filtration through a BioPak filter (Millipore Co.) in order to remove RNase.

All measurements were performed in 10 mM sodium acetate (pH 5.5) or sodium phosphate buffer solution (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. Before measurements, RNA-containing samples were annealed as follows: heating at 85°C for 10 min, and gradually cooling to 5°C (3°C/min). Then the solution temperature was gradually raised to 20°C (1.0°C/min). Typically, the probe was added to the annealed RNA-containing solution, and measurements were performed after a 30 min incubation at 25°C.

UV-Vis and fluorescence spectroscopic measurements

Absorption and fluorescence spectra were recorded on 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 2 × 10 mm quartz cell (optical path length: 10 mm) and a 3 × 3 mm quartz cell, respectively. Fluorescence quantum yield (Φ) was determined relative to fluorescein,[S1,S2] in which COT probe in free or target siRNA-bound state was examined ([COT probe] = 1.0 μM, [target siRNA] = 6.0 μM).

Determination of fluorometric binding constants by fluorescence titration experiments

Fluorescence titration experiments were performed as previously described.[S2,S3] The probe concentration was fixed at 500 nM and the concentration of the target siRNA was varied from 0 to 6.0 μM. The changes in fluorescence intensity of the probe at the maximum wavelength (534 nm) were monitored as a function of the concentration of the target siRNA. The resulting titration curves were analyzed by nonlinear least-squares regression based on a 1:1 binding fitting model:

$$F = 0.5 \times \varepsilon \times [(C_{\text{probe}} + C_{\text{RNA}} + K_{\text{fl}}) \pm \{(C_{\text{probe}} + C_{\text{RNA}} + K_{\text{fl}})^2 - 4 \cdot C_{\text{probe}} \cdot C_{\text{RNA}}\}^{0.5}]$$

K_{fl} is the fluorometric binding constant for the probe to the target siRNA. [probe], [RNA], and [complex] are the concentrations of the probe, siRNA, and probe-siRNA complex, respectively. C_{probe} (= [probe] + [complex]), C_{RNA} (= [RNA] + [complex]), and C_{complex} are the total concentrations of the probe, siRNA, and

probe-siRNA complex, respectively. *F* is the observed fluorescence intensity of the probe in the presence of the target RNA. ε is the conversion coefficient, which is defined as *F*/μM. This coefficient allows the conversion of the fluorescence data to binding data assuming that maximum fluorescence intensity corresponds to 100% complexation because the probe itself shows negligible fluorescence in the absence of siRNA. All data analyses were done using KaleidaGraph software 4.0 (Synergy Software, Reading, PA).

UV melting experiments

UV melting experiments were conducted using a UV-Vis spectrophotometer Model UV-2450 (Shimadzu Co. Ltd., Kyoto, Japan) equipped with a thermoelectrically temperature-controlled micro-multicell holder (8 cells, optical path length: 10 mm). Before each experiment, the sample solutions containing annealed RNA and the probe were put into the cells and covered with paraffin oil and silicon caps, and then cells were incubated for 3 h at 20°C. Absorbance at 300 nm was recorded while increasing temperature from 10 to 100°C with a rate of 0.5°C /min and then corrected by subtracting the absorbance at 370 nm. T_m values were determined from a differential method.

CD measurements

CD spectra were measured at room temperature with a JASCO model J-800 spectropolarimeter (Japan Spectroscopic Co. Ltd.) using a micro sampling disk (optical path length: 1 mm). Scan rate was set to 50 nm/min. Data were accumulated four times.

Stopped-flow measurements

Experiments were performed under second-order conditions on a stopped-flow spectrophotometer system, RSP-2000 (Unisok, Co. Ltd., Osaka, Japan) equipped with a temperature-controlled reaction chamber connected with an F12 refrigerated/heating circulator (Julabo, Seelbach, Germany) as previously described.[S4] Light was collected from a 75W xenon arc lamp housing equipped with a MD200 monochromator (Unisok) to the reaction chamber through a bundled optical fiber. For the measurements, both siRNA and the probe buffer solutions (3.0 μM) were set in each chamber and thermally equilibrated at 25°C. Soon after equilibration, an equal volume (~60 μL) of the two solutions was mixed in the reaction chamber, progress of the reactions was followed by monitoring the decrease in the absorbance at 260 nm. A baseline correction was performed by measuring the absorbance of the buffer prior to the measurements. Optical path length was 10 mm. The second-order association rate constant (k_{on} / $M^{-1}s^{-1}$) was determined from a nonlinear least-square regression by Unispek (Unisok). The dissociation rate constant (k_{off} / s^{-1}) was calculated from $k_{off} = K_d \times k_{on}$.

Solid-phase synthesis of the probes

The probes were manually synthesized by a divergent solid-phase synthesis using commercially available Fmoc/Bhoc PNA monomers and Fmoc-aeg(alloc)-OH which was synthesized previously.[S3] NovaSyn TGR

resin was used as a support. Coupling reagents for PNA monomer and amino acid lysine were COMU and DIEA. The assembly on the resin was performed by repetitive cycles of Fmoc deprotecting with 20% piperidine and 5% DBU in DMF (15 min), coupling with COMU/DIEA in NMP (30 min), and capping with 5% acetic anhydride/6% lutidine in DMF (2 × 5 min). The completion of the coupling reactions was checked by the Kaiser test. Fmoc-aeg(alloc)-OH was utilized for introducing the TO base surrogate. In order to increase solubility, two lysines were introduced to the C-termini of all probes. After completion of the elongation of all monomer units, the alloc group was selectively deprotected with 10 equimolar dimethylamine borane and an equimolar tetrakis (triphenylphosphine) palladium (0) in dichloromethane (2 × 1 h). Then, TO-C_1-COOH[S3] was coupled with the resulting free secondary amine by using TOTU/PPTS/NMM coupling chemistry. After deprotection of the terminal Fmoc group, deprotection of Bhoc groups in the PNA nucleotides and cleavage from the resin were carried out using a mixture of trifluoroacetic acid (TFA)/*m*-cresol (85/15). The solution was filtered for removal of the resin. The resin was washed with a small amount of TFA. Cold diethyl ether was added to the combined filtrate in order to precipitate the crude product. The crude product was purified and analyzed by a reverse-phase HPLC system (pump, PU-2086 Plus x2; mixer, MX 2080-32; column oven, CO-1565 (55°C); detector, UV-2070 plus and UV-1570M (Japan Spectroscopic Co. Ltd., Tokyo, Japan)) equipped with a C18 column (Inertsil ODS3: GL Sciences Inc., Tokyo, Japan) using a gradient of water/acetonitrile containing 0.3% TFA. All probes were verified by MADLI-TOF-MS (4800 Plus MALDI TOF/TOF analyzer: AB Sciex, Tokyo, Japan). From the HPLC and MS data, the purity of the probes was estimated to be > 96%. The concentration of the probe solutions was determined by measuring the absorbance at 260 nm in Milli-Q water at 85°C, with the extinction coefficients of 8600 $M^{-1}cm^{-1}$ for thymine, 6600 $M^{-1}cm^{-1}$ for cytosine, and 9400 $M^{-1}s^{-1}$ for TO.[S5,S6]

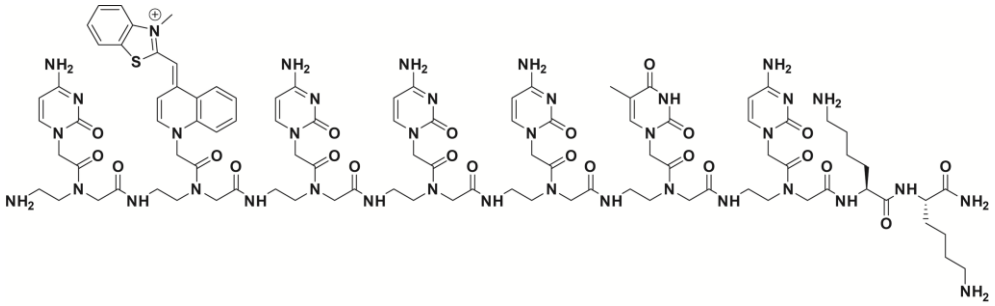

Fig. S1 Chemical structure of the control probe.

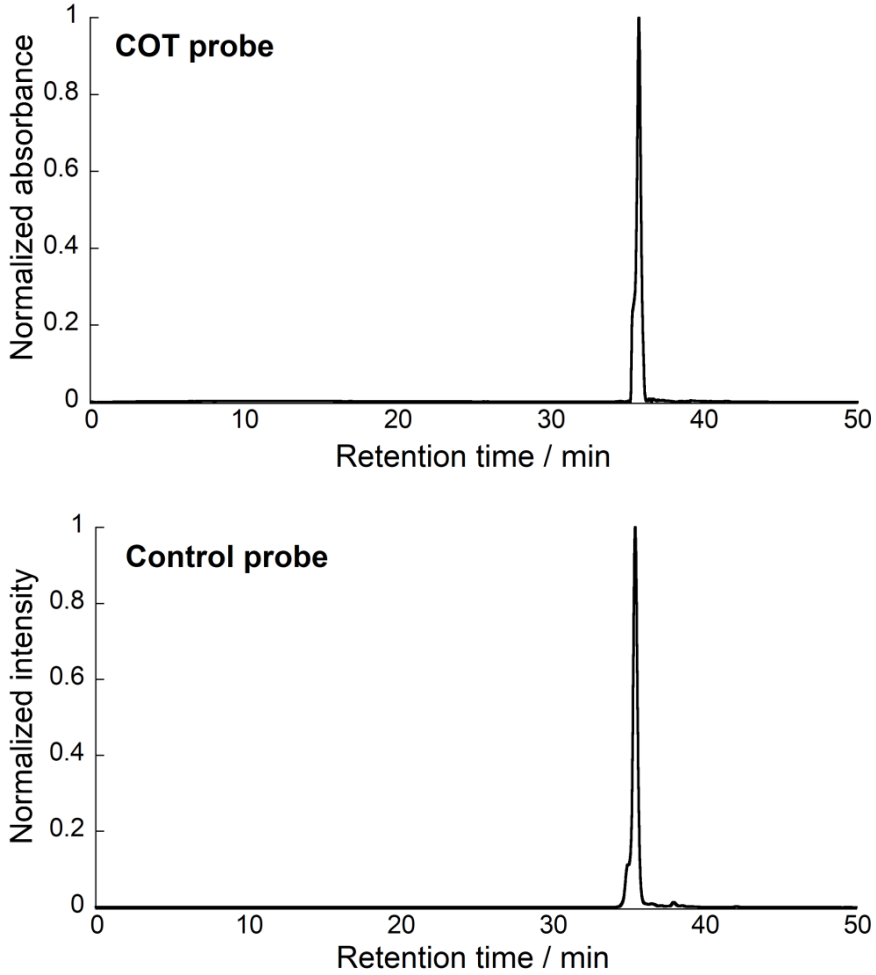

Fig. S2 Analytical HPLC traces of the probes. Absorbance was monitored at 509 nm. Gradient conditions: 0-40% CH_3CN (0.3% TFA) in H_2O (0.3% TFA) during 50 min.

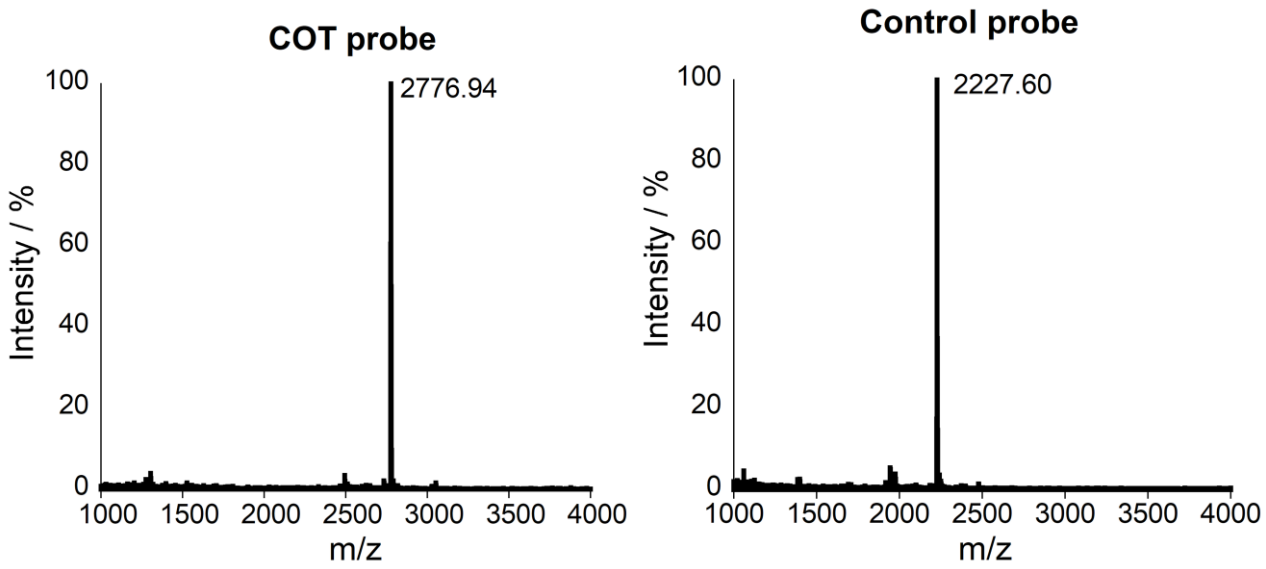

Fig. S3 MALDI-TOF-MS spectra of the purified probes.

Table S1 Probe characterization

Probe	Molecular Formula	Calculated MW ($[M]^+$)	Observed MW ($[M]^+$ or $[M + H]^+$)
COT probe	$C_{119}H_{155}N_{52}O_{27}S^+$	2776.21	2776.94
Control probe	$C_{97}H_{129}N_{38}O_{23}S^+$	2226.98	2227.60

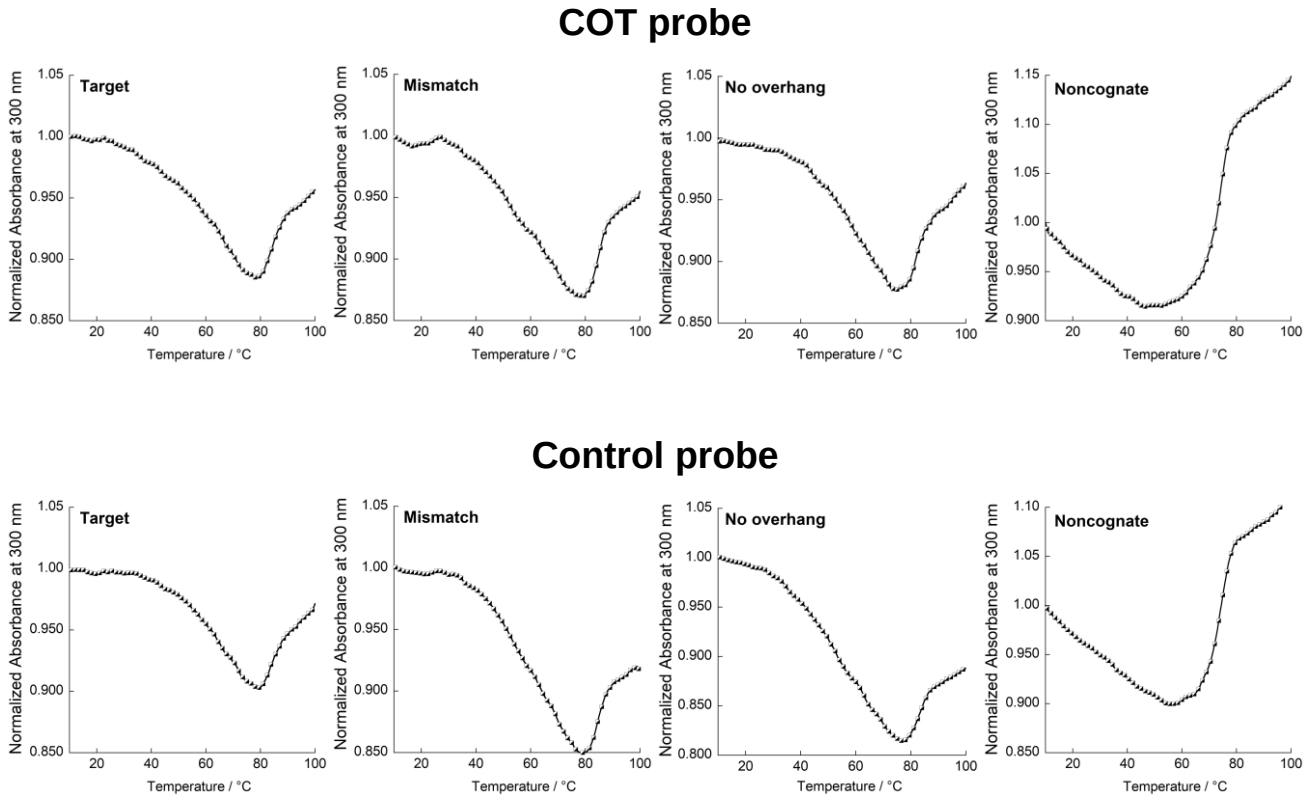

Fig. S4 UV melting curves of the probes (5 μM) in the presence of siRNAs (5 μM) recorded at 300 nm at pH 5.5 (sodium acetate buffer).

Table S2 T_m values of triplexes between the probes and siRNAs, obtained from UV melting experiments (cf. Fig. S4)

T_m* / °C	Target	Mismatch	No overhang	Noncognate
COT probe	66 ± 0.47	65 ± 0.94	64 ± 1.0	34 ± 0.82
Control probe	65 ± 0.41	64 ± 0.62	63 ± 0.47	35 ± 0.62

*Errors are standard deviations obtained from three independent experiments.

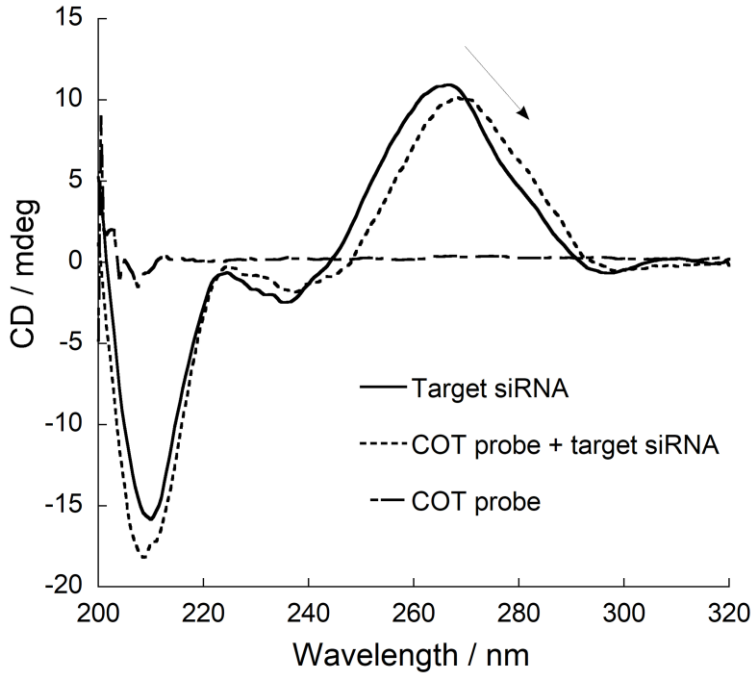

Fig. S5 CD spectral change of target siRNA (10 μM) upon addition of the COT probe (10 μM). The CD peak at around 260 nm was decreased and red-shifted as indicated by arrow. This result is consistent with the previous reports.[S2,S7]

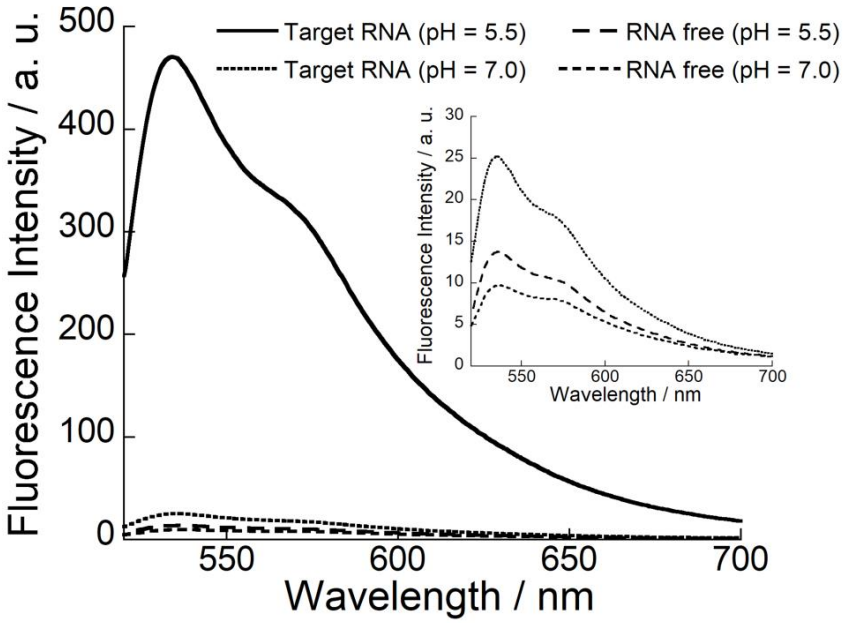

Fig. S6. Fluorescence spectra of the COT probe (1.0 μM) in the absence and presence of target siRNA (1.0 μM) at pH 5.5 (sodium acetate buffer) and pH 7.0 (sodium phosphate buffer).

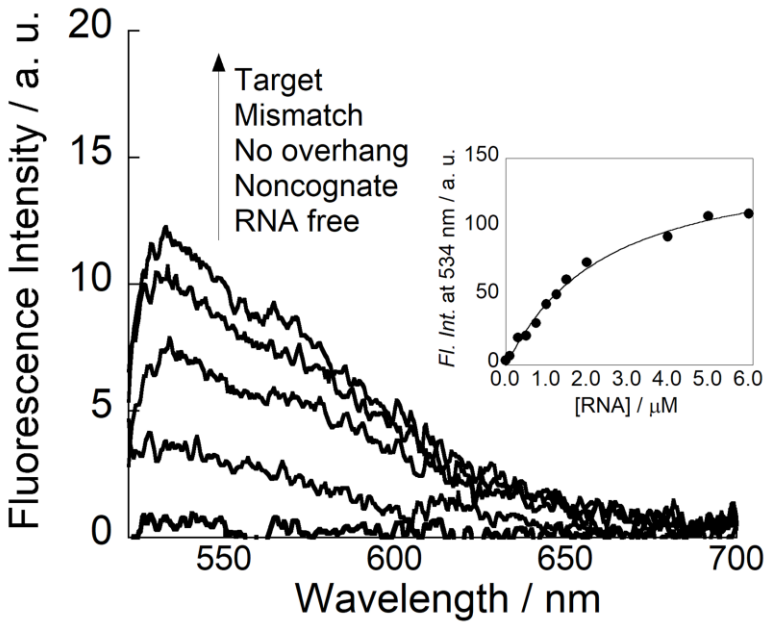

Fig. S7 Fluorescence spectra of the control probe (100 nM) in the absence and presence of siRNAs (100 nM) at pH 5.5. Inset: Fluorescence titration curve for the binding of the control probe (500 nM) to target siRNA (0–6.0 μM) at pH 5.5. Excitation: 509 nm. Analysis: 534 nm. Temperature, 25 °C.

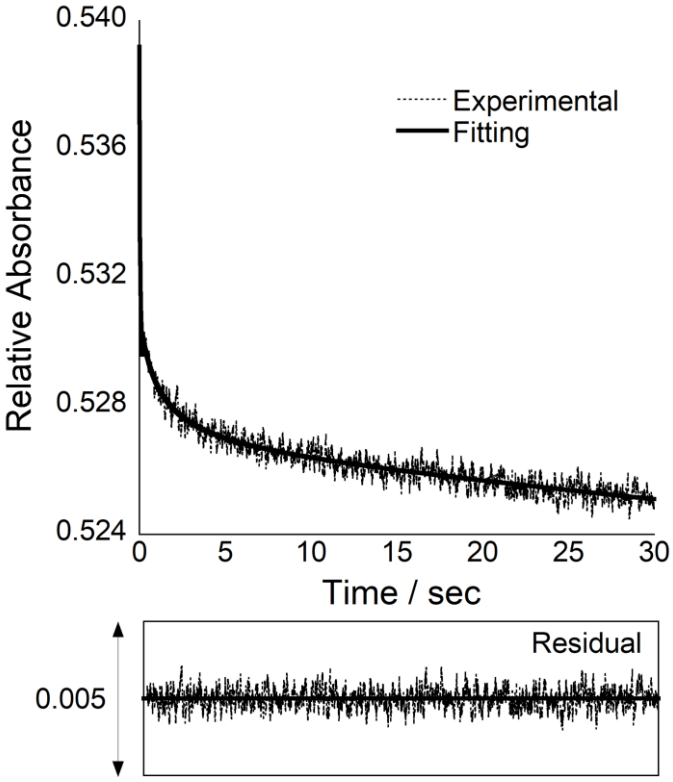

Fig. S8 Stopped-flow kinetics trace for the control probe (3.0 μM) binding to equimolar target siRNA at 25°C. The fitting curve is the bold line, and the corresponding residual plot is presented below the kinetics trace. k_{on} value was determined as 5.2 ± 0.45 × 10^5 M^{-1} s^{-1}. k_{off} value was calculated as 1.2 ± 0.21 s^{-1}.

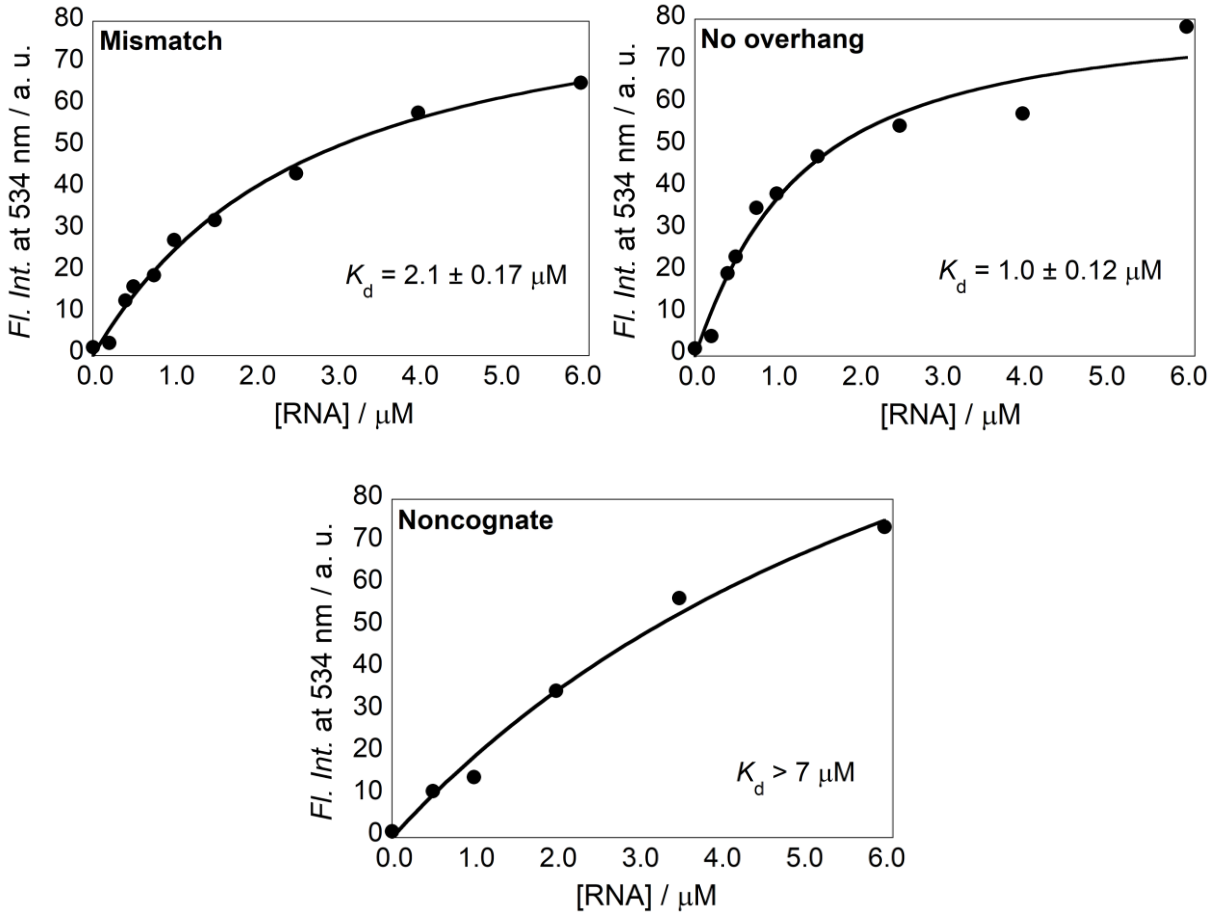

Fig. S9 Fluorescence titration curves for the binding of COT probe (500 nM) to control siRNAs (0–6.0 μM) at pH 5.5. Excitation: 509 nm. Analysis: 534 nm. Temperature, 25 °C.

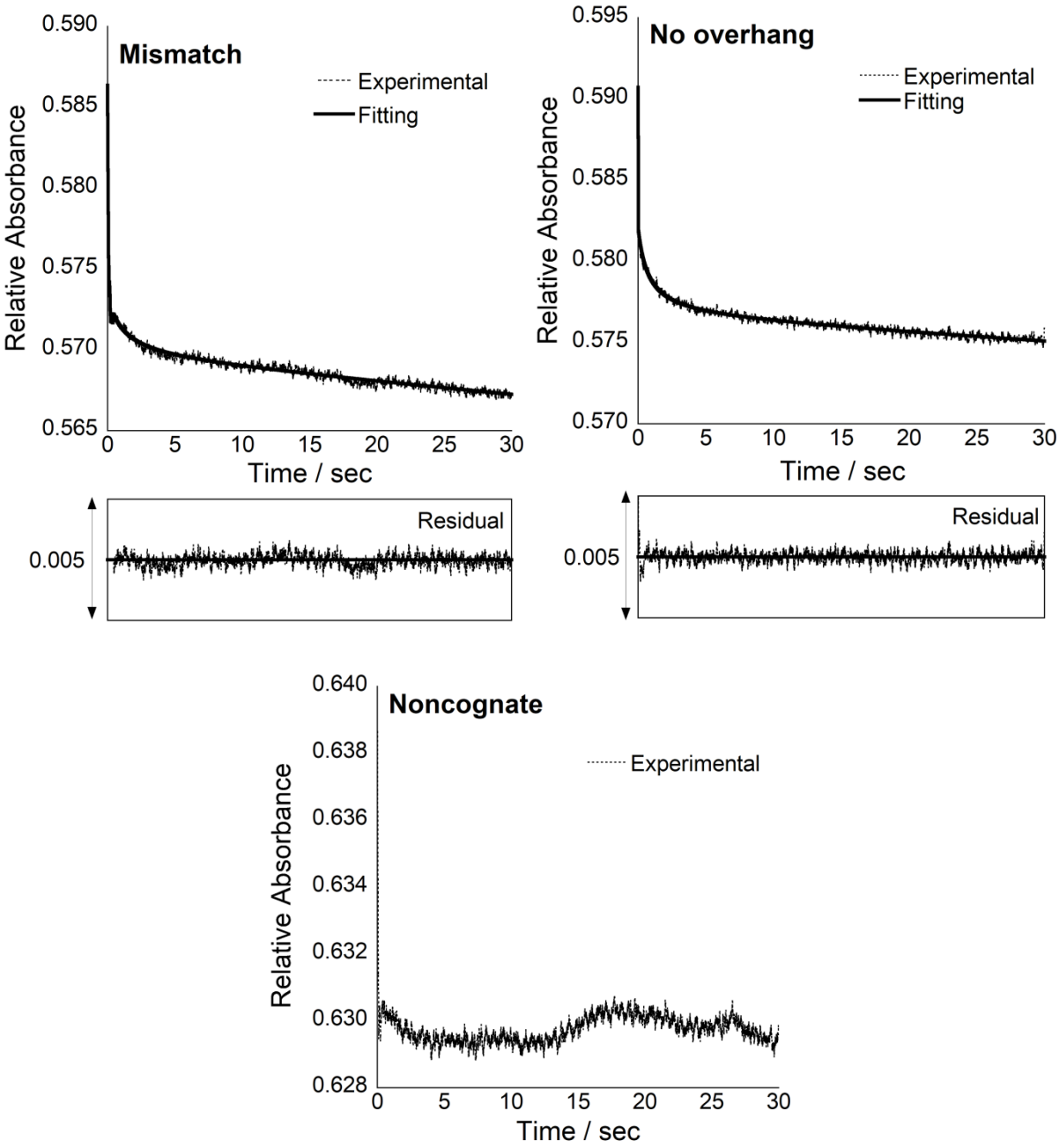

Fig. S10 Stopped-flow kinetics traces for the COT probe (3.0 μM) binding to equimolar control siRNAs at 25°C. The fitting curve is the bold line, and the corresponding residual plot is presented below the kinetics trace. As for noncognate siRNA, we observed little change in the absorbance due to very weak binding with COT probe.

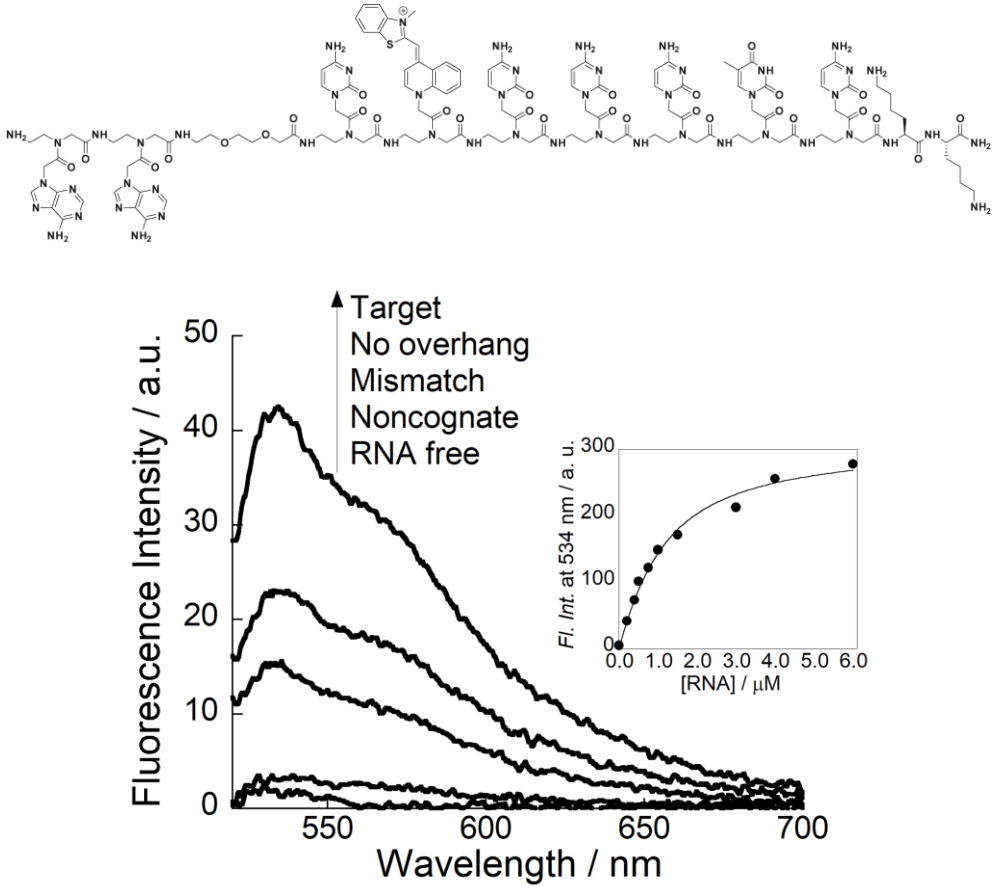

Fig. S11 Effect of spacer introduction between overhang recognition unit and tFIT unit in COT probe on the fluorescence response for siRNAs.

As a preliminary experiment, we examined an 2-(aminoethoxy)ethoxyacetic acid (AEEA) spacer, considering the optimized spacer length in the case of triplex tethered oligonucleotide probes (TOPs).[S8,S9] Fig. S11 shows the fluorescence spectra of the resulting probe (100 nM) in the absence and presence of siRNAs (100 nM) at pH 5.5. While this AEEA spacer-having probe showed the larger response for target siRNA than control siRNAs, its selectivity was inferior to COT probe (cf. Fig. 2 in the main text). In addition, the binding affinity for target siRNA (K_d = 890 ± 68 nM) was found to be weaker than that of COT probe from the fluorescence titration experiments (inset of Fig. S11). Accordingly, it is needed to optimize the spacer length in COT probe by the systematic examination in order to enhance the binding ability for target siRNA. Also, the spacer property like hydrophobicity (hydrophilicity) would affect the probe functions.

Table S3 Comparison of the K_d values of the probes for target siRNAs

	Py-AA-TO**	COT probe	Control probe	AEEA spacer-having probe
K_d* / μM	3.5 ± 0.40	0.34 ± 0.043	2.3 ± 0.36	0.89 ± 0.068

* Errors are standard deviations obtained from three independent experiments.

** The value is from the literature (Ref. 7a in the main text)

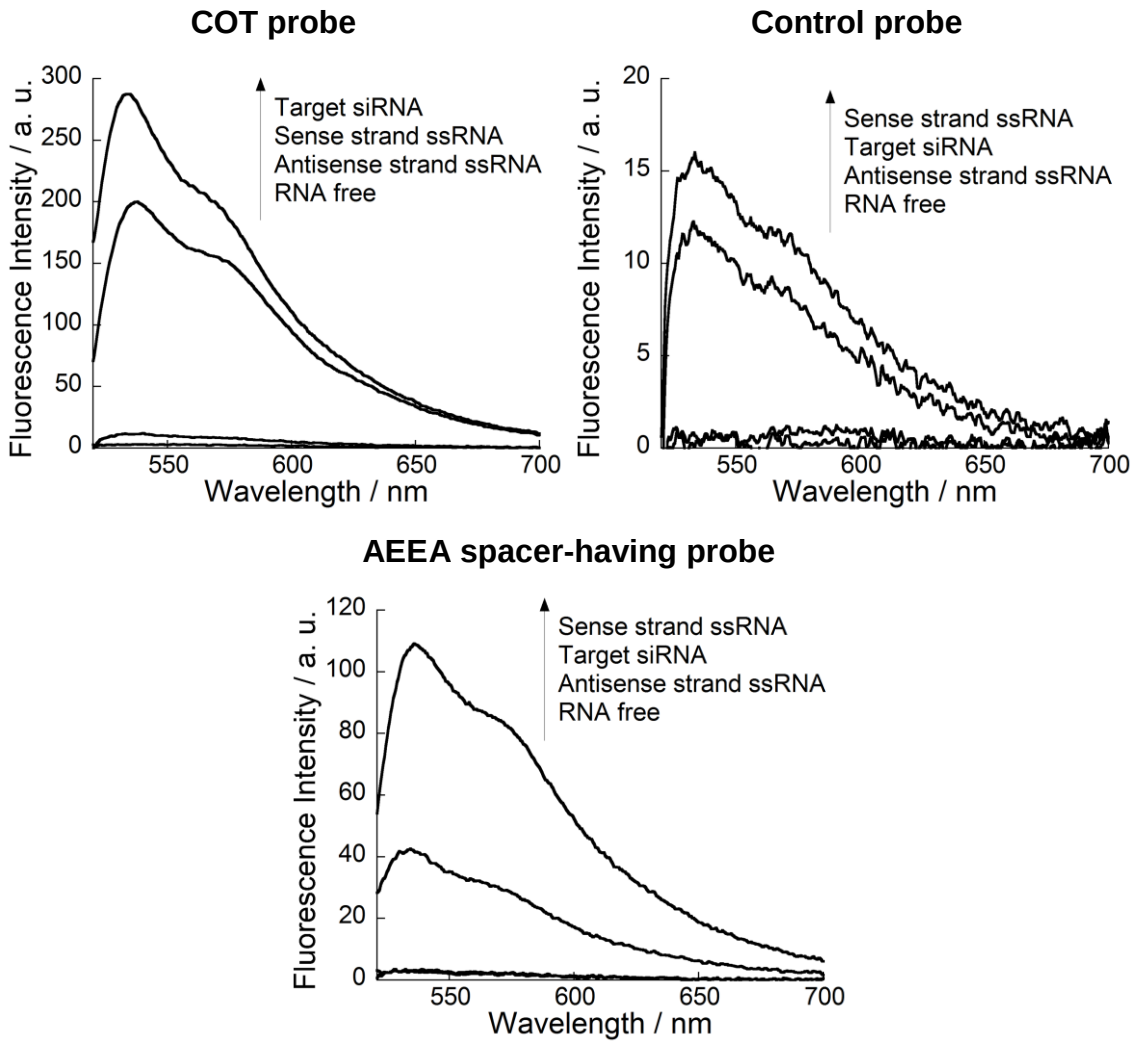

Fig. S12 Fluorescence response of the probes (100 nM) to single-stranded RNAs (ssRNAs: 100 nM) at pH 5.5. Sequences of ssRNAs were shown in Fig. 1C in the main text. For comparison, the response to target siRNA was also shown.

We found that all probes showed the light-up response for sense strand ssRNA whereas very weak response was observed for antisense strand ssRNA. This would be due to the possible duplex formation with sense strand ssRNA in a parallel orientation, as observed for the parent tFIT probes.[S3] In contrast to control probe and AEEA spacer-having probe, COT probe still retained target siRNA selectivity under the present condition. Thus, it is likely that the overhang recognition unit in COT probe favorably contributes to the observed selectivity for target siRNA over ssRNAs.

Reference

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