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Supporting information for

Conjugating pyrene onto PNA-based fluorescent probes for improved detection selectivity toward double-stranded siRNA

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Experimental

Reagents: All of RNAs were custom synthesized and HPLC purified 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. MCF-7 cells were kindly provided by Dr. Kosuke Ino (Tohoku University). Water was deionized (> 18.2 MΩcm 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 at 5°C (3°C/min). Then the solution temperature was gradually raised to 25°C (1.0°C/min). Errors are the standard deviations obtained from three independent experiments ($N = 3$).

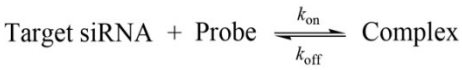
Fluorescence measurements: Fluorescence spectra were recorded on a JASCO FP-6500 spectrofluorophotometer with a thermoelectrically temperature-controlled cell holder (Japan Spectroscopic Co. Ltd., Tokyo, Japan). Measurements of fluorescence spectra were done using a 3 × 3 mm quartz cell. Fluorescence quantum yield (Φ) was determined relative to fluorescein,[S1] in which the probe in the absence or presence of target siRNA was examined ([Probe] =1.0 μM, [target siRNA] = 6.0 μM).

Melting temperature experiments: Melting temperature 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) according to our previous report (ref 7 in the main text).

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). 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.

Here, we consider the biomolecular reaction between the probe and target siRNA as shown in the following equation:



where k_{on} and k_{off} are the association and dissociation rate constants, respectively. As described in our previous report (ref. 29 in the main text), k_{on} value was estimated based on the absorbace change at 260 nm by a nonlinear least-square regression provided by Unispek (Unisok). In this analysis, k_{on} is assumed to be much larger than k_{off}, as typically seen in the triplex formation events. This allows us to quantitatively determine k_{on} value. On the other hand, the dissociation rate constant (k_{off} / s^{-1}) was calculated from $k_{off} = K_d \times k_{on}$.

Probe synthesis: All probes were synthesized by solid-phase synthesis using a microwave reactor (Biotage Initiator, Biotage, Uppsala, Sweden) based on Fmoc/Bhoc chemistry, with a Rink-Amide-Chem Matrix resin (Biotage). The crude product was purified by a reverse-phase HPLC system (pump, PU-2086 Plus ×2; mixer, MX 2080-32; column oven, CO-1565; detector, UV-2070 plus and UV-1570M (Japan Spectroscopic Co. Ltd.)) equipped with a C18 column (Inertsil ODS3; GL Sciences Inc., Tokyo, Japan) using a gradient of water/acetonitrile containing 0.3% trifluoroacetic acid (TFA). All probes were verified by MADLI-TOF-MS (Bruker Daltonics autoflex Speed-S1, Germany).

Detection of siRNA in the samples containing total RNAs: Total RNAs were extracted from MCF-7 cells using Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA, U.S.A.) according to the manufacturer's protocol. After different concentrations of siRNAs were spiked into total RNA (825 ng), PyGly1 and buffer solutions were added. Fluorescence spectra of the resulting mixture solution (100 μL) were measured by FP-6500 spectrofluorophotometer.

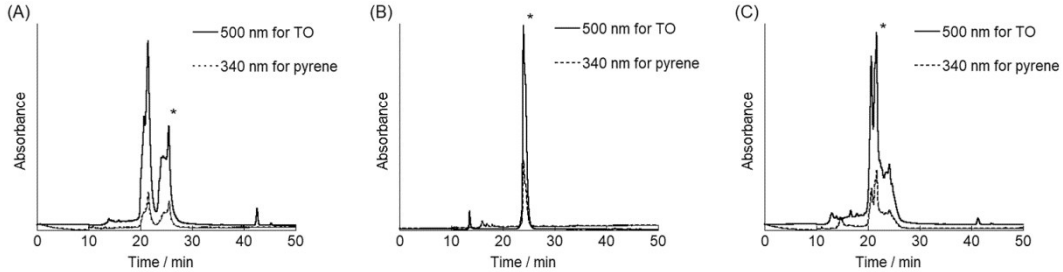


Fig. S1 HPLC profiles for the purification of (A) Py-Gly0, (B) Py-Gly1 and (C) Py-Gly2. Gradient condition: 20-40% CH_3CN (0.3% TFA) in H_2O (0.3% TFA) during 50 min. Absorbance at 500 nm and 340 nm for TO and pyrene units was monitored. The peak (*) was collected and identified as the purified probe by MALDI-TOF-MS (Figure S2).

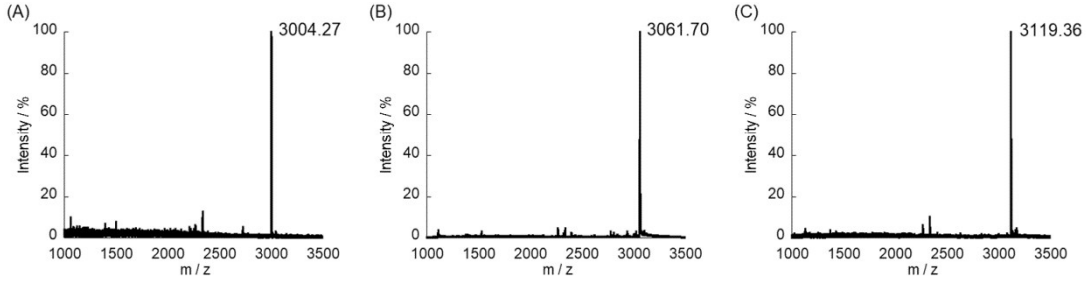


Fig. S2 MALDI-TOF-MS spectra of the purified probes (A: Py-Gly0, B: Py-Gly1, C: Py-Gly2).

Table S1 Probe characterization

Probe	Observed mass (m/z)	Calculated mass ([M^+])	Retention time (min)
Py-Gly0	3004.27	3004.26	25.5
Py-Gly1	3061.70	3061.29	24.0
Py-Gly2	3119.36	3120.32	22.0

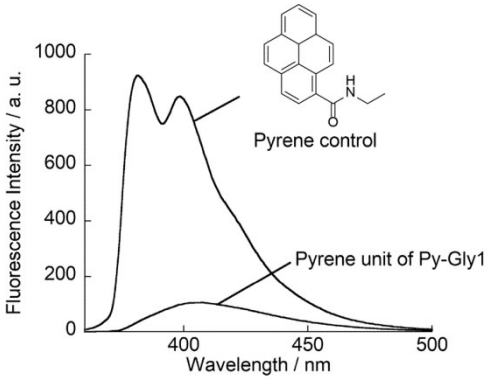


Fig. S3 Fluorescence spectra of the pyrene unit in Py-Gly1 (3.0 μM) and a pyrene control (3.0 μM). Measurements were done in pH 5.5 buffer.

The pyrene unit of Py-Gly1 shows weaker emission than the pyrene control. In addition, the vibronic structure is not observed for Py-Gly1. These results indicate the possible intramolecular interaction between the pyrene unit and PNA or TO unit in Py-Gly1.

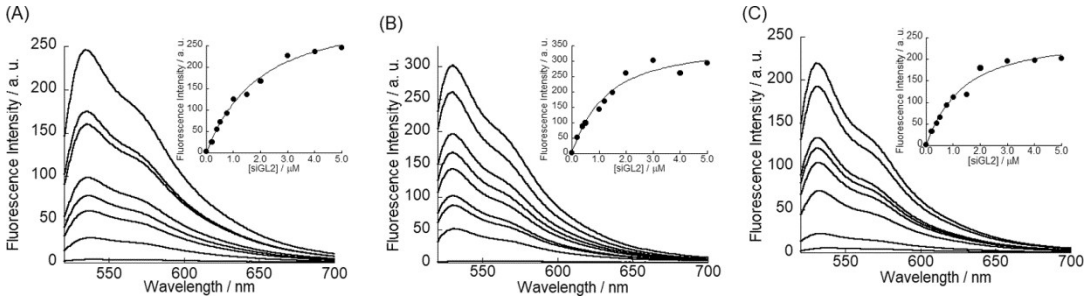


Fig. S4 Fluorescence titration curves for the binding of the probes (500 nM: (A) Py-Gly0, (B) Py-Gly1, (C) Py-Gly2) to target siRNA (0-5000 nM) at pH 5.5. Temperature, 25°C. Excitation, 509 nm. Analysis, 534 nm.

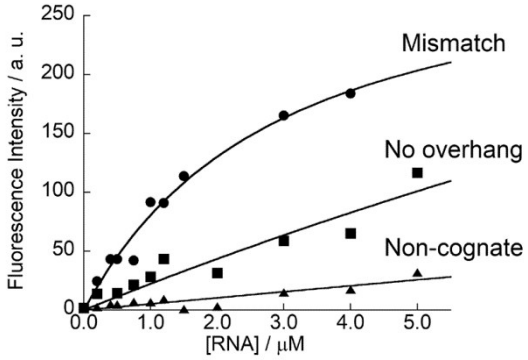


Fig. S5 Fluorescence titration curves for the binding of Py-Gly1 to control double-stranded siRNAs. Measurement conditions are the same as those given in Fig. S3.

K_d values for mismatch and no overhang siRNAs were determined as 1.8 ± 0.65 and 7.4 ± 0.92 μM (N = 3) whereas the binding affinity for non-cognate siRNA was very weak (K_d > 20 μM). Thus, Py-Gly1 showed the stronger binding affinity for target siRNA (K_d = 1.1 ± 0.10 μM) compared to these control siRNAs.

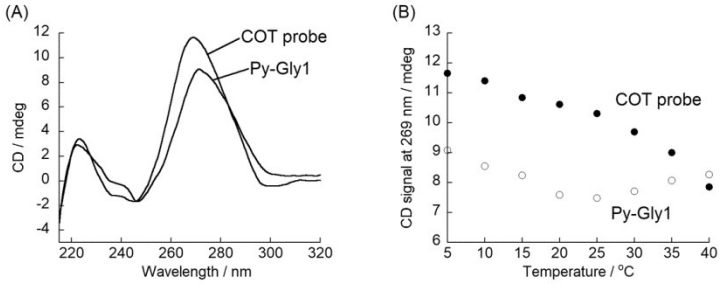


Fig S6 (A) CD spectra of single-stranded sense strand in the presence of COT probe or Py-Gly1 at 25°C in pH 5.5 buffer. (B) CD signal at 269 nm at various temperatures.

Table S2 Dissociation constants, and kinetic parameters of the binding of the probes to target siRNA at 25°C in pH 5.5 buffer.[a]

	K_d / μM	k_{on} /10^6 $M^{-1}\cdot s^{-1}$	k_{off} / s^{-1} [b]
Py-Gly1	1.1 ± 0.10	0.50 ± 0.24	0.55 ± 0.26
COT[c]	0.34 ± 0.043	3.0 ± 0.31	1.0 ± 0.17
control[c]	2.3 ± 0.36	0.52 ± 0.045	1.2 ± 0.21

[a]Error bars are the standard deviations obtained from three independent experiments. [b]k_{off} values were calculated from $k_{off} = K_d \times k_{on}$. [c]Values are from the literature (Ref. 7 in the main text).

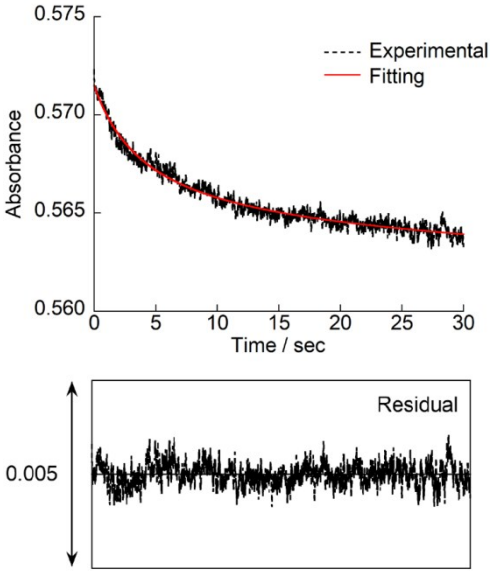


Fig. S7 Stopped-flow kinetics trace for the binding of Py-Gly1 (3.0 μM) to equimolar target siRNA at 25°C in pH 5.5 buffer. The fitting curve is the bold line and the corresponding residual plot is presented below the kinetics trace.

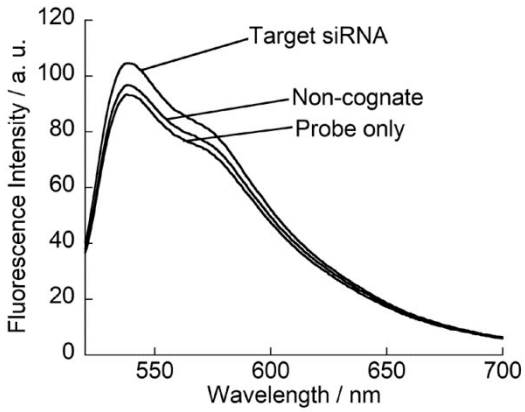


Fig. S8 Fluorescence spectra of PyGly1 (1.0 μM) in the absence and presence of total RNAs (825 ng) extracted from MCF7 cells after transfection of siRNAs (3.0 μM: target or non-cognate) by jetPRIME^{TM}. Measurements were done in pH 5.5 buffer. Temperature, 25°C. Excitation, 513 nm.

Reference

S1. D. V. Jarikote, N. Krebs, S. Tannert, B. Röder and O. Seitz, *Chem. Eur. J.*, 2007, **13**, 300–310.