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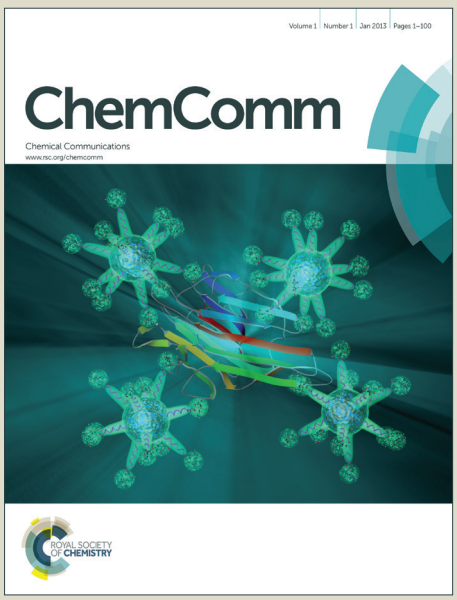





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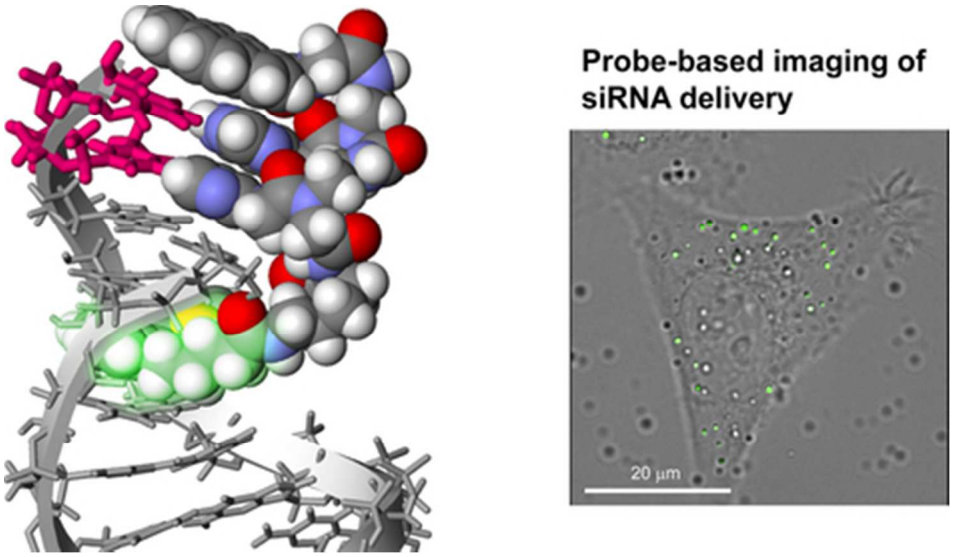


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ARTICLE TYPE

# Synthetic Fluorescent Probes Capable of Selective Recognition of 3'-Overhanging Nucleotides for siRNA Delivery Imaging

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**Peptide nucleic acid (PNA)-thiazole orange (TO) conjugates are developed as fluorescent probes capable of selective recognition of 3'-overhanging nucleotides of siRNAs for accurate analysis of the siRNA delivery process.**

Small interfering RNAs (siRNAs) have been widely used for the study of gene functions due to their silencing abilities in a sequence-specific manner through an RNA interference (RNAi) process.[1] The siRNAs also hold great potential as therapeutic agents for the treatment of various diseases. However, practical implementation of siRNA-based therapy faces several challenges,[2] of which one of major barriers is to design safe, efficient, and specific delivery systems of the siRNAs to target cells or organs.[3] Much attention has been thus paid to the development of non-viral carriers mainly based on polymers or lipids.[4] For the assessment of these carriers in details, it should be of great importance to analyze the siRNA delivery process, such as cellular uptake and release the siRNAs from the carriers at the molecular level.

In this context, fluorescently labeling of siRNAs is commonly used, where organic fluorophores[5] or fluorescent nanoparticles[6] are covalently labeled at the termini of the siRNAs. Also, siRNAs modified with fluorescent nucleotides[7] or nucleotide surrogates[8] have been recently developed. These methods are useful for fluorescence imaging of siRNAs in the living cells, which enabled to probe the cellular uptake using delivery carriers by transfection as well as the intracellular trafficking of the siRNAs. However, the need to fluorescently labeling or modification to the target siRNAs presents a potential drawback to reduce their intrinsic gene silencing properties since the fluorophores labelled or modified with the siRNAs can perturb the entry of the siRNAs to RNAi pathway.[9] Hence, it is needed to carefully consider the position and the number of the fluorophores in the siRNA sequences so as not to lose gene silencing activities of target siRNAs.

In this work, we report on a new strategy for analysis of siRNA delivery without relying on fluorophore labeling and modification of siRNAs, for which we focused on fluorescent probes capable of non-covalently binding to the siRNAs. This class of probes are expected as affinity-labeling agents suitable for fluorescence imaging of the siRNA delivery process. In addition, we envision no reduction in gene silencing activity of the siRNAs due to the possible dissociation of the probes from the siRNAs when target siRNAs were incorporated into RNAi pathway. The approach

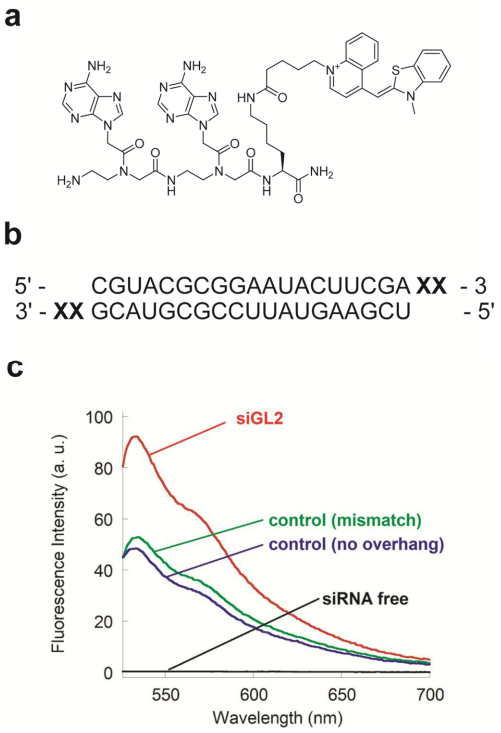


Figure 1. Design of peptide nucleic acid (PNA) conjugated with thiazole orange (TO) for selective recognition of 3'-overhanging nucleotides of the siRNAs. (a) Chemical structure of **AA-TO**. (b) Sequences of siRNAs against firefly luciferase GL2 gene (**siGL2**: X = dT) and control siRNAs having no overhangs (X = none) or overhanging nucleotides that mismatched with the PNA units of the probe (X = dA). (c) Fluorescence spectra of **AA-TO** (200 nM) in the absence and presence of target siRNAs (20 nM) in phosphate buffer (*I* = 0.06 M, pH 7.0) at 20°C. Excitation: 514 nm.

using RNA-binding fluorescent probes was previously reported, where RNA intercalators were utilized for the analysis of cellular uptake of siRNAs.[10] However, these intercalators are highly likely to show little binding selectivity for the target siRNAs over other intracellular nucleic acids since they simply aim to target the double helices.[11] Non-selective binding of the intercalators would result in substantial background fluorescence and then failure to get selective visualization of target siRNAs inside cells. In contrast, we aimed to develop fluorescence probes possessing selectivity to target siRNAs and, as such, our probes are designed to specifically recognize both 2-nt overhanging nucleotides on the

3'-ends and the double-helix region near the overhangs in the target siRNAs. Here, peptide nucleic acids (PNAs)[12] were conjugated with thiazole orange (TO),[13] as shown in Fig. 1a. PNA was utilized as a recognition unit of the 3'-overhanging nucleotides of the target siRNAs due to its strong binding to RNAs by virtue of the lack of electrostatic repulsion and good resistance to enzymatic degradation.[12b] While the binding of PNA units with an ultra-short length seems very weak, coaxial stacking with the 5'-terminal nucleotide of siRNAs is expected to stabilize this binding event.[14] The binding can be further stabilized by the intercalation of the TO unit to the double-helix region near the overhanging structure in the target siRNAs, which accompanies the light-up response of the TO unit.[13] Here, PNA-TO conjugates were designed for the target siRNA against firefly luciferase GL2 gene (**siGL2**, Fig. 1b).[15] Then, two consecutive PNA adenines are chosen so as to form the complementary base-pairing with the overhanging deoxyribothymines (dTdT) in **siGL2**, which affords **AA-TO** (Fig. 1a).

We examined the fluorescence response of **AA-TO** (200 nM) to the target **siGL2** (20 nM) at 20°C in phosphate buffer ($I$ = 0.06 M, pH 7.0) (Fig. 1c). In the absence of siRNAs, fluorescence of the TO unit is almost negligible by nonradiative energy loss due to free rotation of the benzothiazole and quinoline rings.[13a] Such negligible background fluorescence in the TO unit is clearly advantageous for the light-up probes and is quite characteristic compared to TO-tethered 10mer PNA oligonucleotide probes.[16] This is highly likely due to no folding-back of the TO unit to interact with ultrashort PNA unit in the case of our probe. The addition of **siGL2** causes the fluorescence enhancement of the TO unit, indicating the intercalation of the TO unit into the double-helix region of **siGL2**. This is supported by the observation of a slight increase in the absorbance and a redshift in the UV-visible absorption spectrum of **AA-TO** with **siGL2** (Fig. S1, ESI†). Notably, the light-up response for **siGL2** was more significant than responses for control siRNAs having no overhanging nucleotides or deoxyriboadenine overhangs that mismatched with the PNA units of **AA-TO** (Fig. 1b). Thus, **AA-TO** selectively recognizes the dTdT overhang in **siGL2**. The screening of the spacer length between PNA and TO units led to **AA-TO** being the best candidate, although all conjugates showed selective fluorescence responses for **siGL2** (Fig. S2, ESI†). This selectivity arises from the possible formation of complementary base-pairing between PNA adenines of **AA-TO** and the dTdT overhang of **siGL2**, as clarified by the comparison with a control compound, TO derivative that lacks PNA units (Fig. S3, ESI†). The control compound has moderate fluorescence response compared to **AA-TO** and importantly, shows no selectivity for the overhanging nucleotides of the target siRNAs. These results demonstrated PNA-TO conjugate was a promising candidate as a fluorescent probe for analyzing target siRNAs.

However, PNA-TO probes should be further improved regarding the selectivity for overhanging nucleotides so as to enhance the applicability to the analysis of siRNA delivery in living cells. We found the selectivity of **AA-TO** for **siGL2** decreased with increasing concentration of siRNAs and it almost disappeared in the presence of more than an equimolar amount of target siRNAs (Fig. S4, ESI†). We reasoned that this came from non-selective intercalation of the TO unit of **AA-TO** for the double-helix region of the target siRNAs, considering the large responses for control siRNAs. Accordingly, the key to high selectivity for the overhanging nucleotides of the target siRNAs is to suppress such non-selective intercalation. Here, we

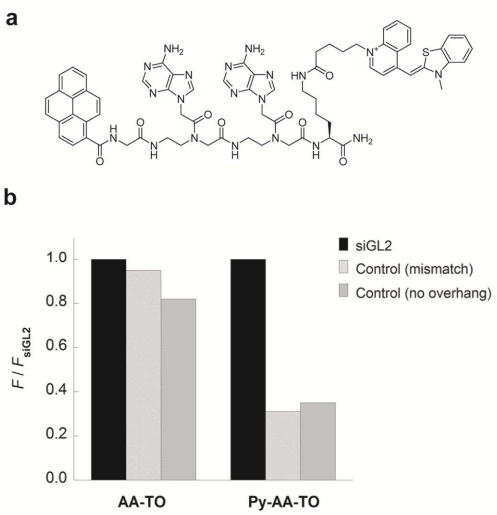


Figure 2. Pyrene-containing probe (**Py-AA-TO**) for improved selectivity for 3'-overhanging nucleotides of the siRNAs. (a) Chemical structure of **Py-AA-TO**. (b) Selectivity of light-up response of the probes (200 nM) for **siGL2** over control RNAs, measured in phosphate buffer ($I$ = 0.06 M, pH 7.0) at 20°C. Concentration of RNAs: 200 nM. Excitation: 514 nm. $F$ and $F_{siGL2}$ denote the fluorescence intensity at 534 nm of the probes in the presence of target RNAs and **siGL2**, respectively.

integrated a pyrene into the N-terminal of **AA-TO** because the high propensity of pyrene for π-stacking can promote the intramolecular stacking of the probe in the absence of target siRNAs. Therefore, non-selective intercalation of the TO unit was expected to be effectively suppressed, as has been successfully demonstrated in nucleotide-acridine[17a] and triaminotriazine-acridine conjugates.[17b]

Actually, we found that the integration of a pyrene unit into **AA-TO** led to the significant improvement of the selectivity for **siGL2** (Fig. 2 and Fig. S5, ESI†). The resulting probe, **Py-AA-TO** (Fig. 2a)**,** showed high selectivity for equimolar amounts of **siGL2** over control RNAs. The light-up response for **siGL2** was nearly three times larger than that for control RNAs whereas **AA-TO** showed little selectivity for **siGL2** under the identical conditions (Fig. 2b and Fig. S6). This selectivity of **Py-AA-TO** was retained even in the presence of excess amounts of target siRNAs (Fig. S7, ESI†). It is highly likely that the improved selectivity for target siRNAs was derived from facile intramolecular stacking between pyrene and TO units of **Py-AA-TO** in the absence of target siRNAs. The formation of such an intramolecular stacking was suggested by the observation of a redshift and a large hypochromic effect in the UV-visible absorption spectra for both pyrene and TO units compared to their control compounds (Figs S8 and S9, ESI†). A Monte Carlo conformational search also revealed an energetically-stable conformer adapting the pyrene-TO stacking in the largest population (Fig. S10, ESI†). On the other hand, the binding of **Py-AA-TO** would result in the unfolding of intramolecular stacking of **Py-AA-TO** for effective intercalation of the TO unit for the target **siGL2** (cf. Fig. 2b). While further structural studies such as NMR analysis are needed to clarify the interactions between pyrene units and target siRNAs, we speculate that a pyrene unit interacts with a 3'-terminal base pair as a molecular cap,[18] as seen in the possible binding mode of **Py-AA-TO**

obtained by molecular modeling (Fig. S11, ESI†). This is consistent with the observation that the vibronic structure of the pyrene unit disappears upon binding to **siGL2** (Fig. S9, ESI†). In addition to high selectivity to the overhanging nucleotides of the target siRNAs, this selectivity of our probes can be rationally controlled by adopting the PNA units according to the Watson-Crick base-pairing rules (Fig. S12, ESI†). The use of PNA cytosines in the probe design led to the development of the probe with selectivety to overhanging deoxyriboguanines (dGdG) of the target siRNAs.

Finally, **Py-AA-TO** was applied as an affinity-labeling agent for siRNA for the fluorescence imaging of siRNA delivery in living cells. Here, **Py-AA-TO**/**siGL2** complex was mixed with jetPRIME™, a polymer-based transfection reagent that was commercially available and the cellular uptake of the resulting **Py-AA-TO**/**siGL2**/carrier polyplexes was monitored by fluorescence microscopy. Some punctuate green fluorescence appeared in the cytoplasm after a 30 min incubation of the polyplex with HeLa cells and this fluorescence signal gradually increased over the course of 3 h (Fig. S13, ESI†); this time course is consistent with a previous report on cellular uptake of similar polyplexes.[19] Our colocalization study using **Py-AA-TO** and **siGL2** modified with fluorophore revealed that the fluorescence emission of **Py-AA-TO** indeed results from the bound state with **siGL2** in the polyplexes (Fig. 3a). In the channel of **siGL2** modified with fluorophore (Fig. 3a, middle), we observed the distribution of fluorescence throughout the cytoplasm, which indicates that some amounts of **siGL2** modified with fluorophore were diffused after the release from the carriers into the cytoplasm. By contrast, **Py-AA-TO** showed only punctate fluorescence, and no distribution of fluorescence throughout the cytoplasm. Thus, **Py-AA-TO** was highly likely to be rapidly dissociated from the complex after the release from the carriers, presumably due to the moderate binding affinity of **Py-AA-TO** to **siGL2** as was seen in other RNA-binding fluorescent probe, FLEth.[10a] Indeed, the dissociation constant ($K_d$) of **Py-AA-TO** to the dTdT overhang in the **siRNAs,** determined as 3.5 ± 0.40 μM ($n$ = 3) from the fluorescence titration experiments (Fig. S14, ESI†), was almost comparable to that of FLEth ($K_d$ = 1.2 and 4.0 μM).[10a] Intriguingly, dissociated **Py-AA-TO** showed negligible fluorescence arising from non-specific binding to various RNAs in the cytoplasm (Fig. 3a), due to weak binding to RNAs without overhanging nucleotides (cf. Fig. 2b). Also, a control experiment in which the cells are treated with only **Py-AA-TO** shows no fluorescence of **Py-AA-TO** (Fig. S15, ESI†), indicating negligible non-specific binding inside the cells. Accordingly, **Py-AA-TO** can selectively visualize the target siRNAs encapsulated in the polymer carries, which enables accurate analysis of cellular uptake of the siRNAs by transfection and their release from the carriers. In contrast, when traditional RNA intercalators such as ethidium bromide (Fig. 3b) and TO (Fig. S16, ESI†) were utilized under the identical conditions, we observed large fluorescence emission from non-specific binding in the cytoplasm as well as in the nucleolus after the release from the carriers. Such large background fluorescence, especially in the cytoplasm where the components of the RNAi machinery are present, would make it difficult to discriminate the target siRNAs in the polyplex from those released from the carriers. Certain amount of background fluorescence was also observed in both cytoplasm and nucleolus in the case of **AA-TO** having lower selectivity to overhanging nucleotides in the siRNAs than **Py-AA-TO** (Fig. S17, ESI†). Therefore, overhang selectivity of **Py-AA-TO** should be essential for selective visualization of the siRNAs in the polyplex.

Importantly, we confirmed that the use of **Py-AA-TO** as an affinity-labeling agent for target siRNAs did not perturb the RNAi activity of **siGL2** (Fig. 4). From the examination of dual luciferase reporter assays, expression of luciferase gene was remarkably inhibited by **siGL2** while no gene silencing was observed by treating with a scrambled siRNA. The silencing activity of **siGL2** was found to be comparable to that in the presence of **Py-AA-TO**. This was highly likely due to that **Py-AA-TO** could be rapidly dissociated from **siGL2** in the cytoplasm before the entry of **siGL2** into RNAi machinery. It should be noted that we observed no cytotoxicity of **Py-AA-TO** with **siGL2** in the polyplex under the experimental condition (Fig. S18, ESI). In addition to polymer-based carriers described above, **Py-AA-TO** is also able to selectively visualize the target siRNAs encapsulated in a lipid-based carrier (Fig. S19a, ESI†). Similar to polymer-based carriers, we observed no reduction in the activity of **siGL2** by using **Py-AA-TO** in the case of lipid-based carriers (Fig. S19b, ESI†). These results indicate our method has good compatibility with various kinds of delivery carriers. Therefore, we concluded **Py-AA-TO** can function as a

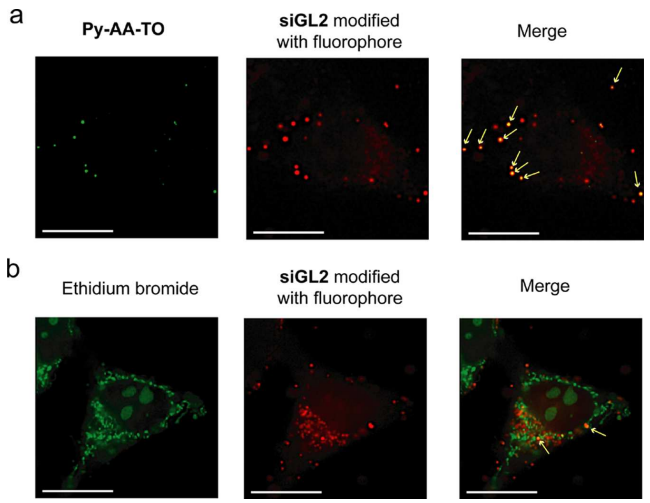


Figure 3. Fluorescence microscope images of HeLa cells after being incubated with the polyplexes containing (a) **Py-AA-TO**/fluorophore (Alexa 647)-modified **siGL2** complex (500 nM) and (b) ethidium bromide/fluorophore (Alexa 647)-modified **siGL2** complex (500 nM) for 3 h. The colocalization of fluorescent probe (green color) and Alexa 647 in siGL2 (red color) apprears as a yellow color, as shown as arrows. Scale bar: 20 μm.

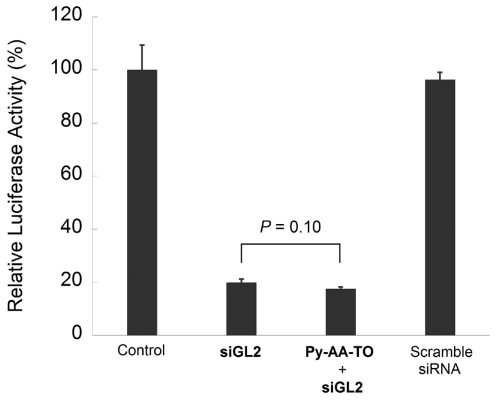


Figure 4. Effect of the use of **Py-AA-TO** as a siRNA affinity-labeling agent on gene silencing activity of **siGL2**. RNAi activity was evaluated using dual luciferace reporter assay system (ESI).

versatile and effective tool for the accurate analysis of the siRNA delivery process by carriers into the living cells.

In summary, we developed PNA-TO conjugates as fluorescent probes capable of selectively recognizing 3'-overhanging nucleotides of siRNAs and applied them as affinity-labeling agents to the analysis of the siRNA delivery process in living cells. To the best of our knowledge, this is the first report on fluorescent probes to selectively bind to overhanging nucleotides of the siRNAs. Significantly, the conjugate having a pyrene unit facilitated accurate analysis of cellular uptake of the siRNAs and their release from the delivery carriers because non-covalent binding of the probe with overhang selectivity enables to selectively visualize the target siRNAs encapsulated in the carriers. This binding nature of our probe results in no reduction in gene silencing activity of the siRNAs. We expect that our fluorescent probes can function as a useful and versatile tool for development of delivery carriers suitable for the practical use of siRNA-based therapy. We also expect that this class of fluorescent probes will be useful for analysis of vector-expressed siRNAs[20] and even endogeneous siRNAs[21] in addition to siRNAs delivered by the carriers in the present study. We are now undertaking further studies in these directions.

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## Notes and references

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† Electronic Supplementary Information (ESI) available: [Experimental details, probe/siRNA interaction analysis, fluorescence imaging using thiazole orange and **AA-TO**, cytotoxicity and gene silencing activity of siRNAs in the presence of the probes]. See DOI: 10.1039/b000000x/

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