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Cellular Uptake of PNA−Terpyridine Conjugates and Its Enhancement by Zn^{2+} Ions

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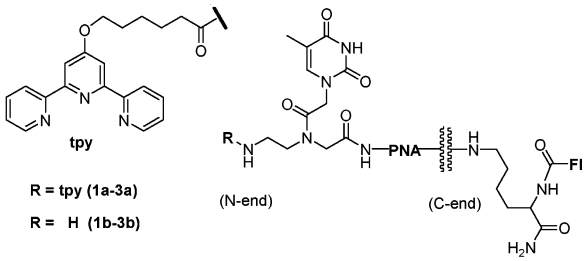
Peptide nucleic acid (PNA) is a DNA mimic with a neutral pseudopeptide backbone.[1] It binds to complementary DNA with high affinity and specificity and displays high biological stability. As such, PNA is an ideal probe for monitoring gene expression on the cellular and molecular level, or it can be applied as an antisense and antigene reagent.

However, fast progress in the applications of PNA has been limited by its poor cellular uptake. Several noninvasive delivery strategies were developed: conjugation to oligopeptides with positively charged side chains,[2] to lipophilic molecules (adamantane,[3] triphenylphosphonium,[4] biotin[5]), to cell-specific receptor ligands,[6] complexation with lipids and a DNA carrier,[7] and backbone modification by introduction of a guanidinium group.[8] Recent critical surveys[9] point out the need for an extended repertoire of simple and effective cellular (and ultimately systemic) delivery methods to optimize performance of PNA-based probes or reagents in an application-dependent manner.

In an effort to control PNA activity chemically, we have recently developed a chelator-modified PNA[10] that increases hybridization after activation with Zn^{2+}, a metal with a high cell-type and tissue-specific distribution. Here we report that chemical modification of PNA by 2,2′:6′,2″-terpyridine (tpy) promotes cellular and nuclear uptake, which is further enhanced by Zn^{2+}.

Solid-phase synthesis of PNAs **1**−**3** (Scheme 1) with randomly chosen sequences (9−20mer, no matching sequences in human genome database), tagged with a C-terminal fluorescent label and N-terminally attached tpy (**1a**−**3a**), is described in the Supporting Information.[11] Three C-terminal lysines were introduced to improve solubility and to facilitate attachment of fluorophores. For comparison, PNA **1c** was prepared, with a C-terminal tetra-Lys tag and N-terminal rhodamine, but lacking tpy. PNA **1a** was selected to study duplex stability with complementary DNA **4** (T_m = 50.1 °C). It is comparable to $(Lys)_3$-modified PNA of the same sequence (T_m = 47.8 °C). The fluorescence emission of PNA **1a** with C-terminally attached dye (even when hybridized to DNA) was 8 times less intense than that of PNA **1c** with the N-terminally attached dye. Reduced fluorescence is likely an effect of conversion of the dye's 2-carboxylic group to the piperidyl amide during PNA synthesis (Supporting Information).[12] Interestingly, PNAs **1a**−**3a** behaved like "molecular beacons",[13] that is, fluorescence increased 3.3−7.0-fold upon hybridization with DNA (Figure 1). Apparently, tpy quenched rhodamine fluorescence, and the tpy−dye contact was disrupted on formation of the rigid PNA/DNA duplex. Fluorescence of **1a**−**3a** was further reduced by addition of 1 equiv of Zn^{2+}. Fluorescence of probes without tpy **1b**−**3b** and **1c** was minimally affected (0.8−1.2-fold, 1.6-fold for hybridization of **2b**) by either hybridization or Zn^{2+} addition. Potentially, hybridization-enhanced fluorescence might be beneficial for the visualization of nucleic acid targets due to reduced background signaling.[13]

Scheme 1. Modified PNA Probes **1**−**3**



PNA 1a (N) tpy-TCACAACTAkk-Fl_1 (C); **PNA 1b** (N) TCACAACTAkk-Fl_1 (C)
PNA 1a', 1a'' (N) tpy-TCACAACTAkk-$Fl_{2,3}$ (C)
PNA 1c (N) Fl_1-TCACAACTAkkkk (C)
PNA 2a (N) tpy-TACACAACTkk-Fl_1 (C); **PNA 2b** (N) TACACAACTkk-Fl_1 (C)
PNA 3a (N) tpy-TCCTCGCCCTTGCTCACCATkk-Fl_1(C)
PNA 3b (N) TCCTCGCCCTTGCTCACCATkk-Fl_1 (C)
Fl_1 = Tetramethylrhodamine, Fl_2 = Nile red, Fl_3 = Coumarin 343,
k = Lysine (C-terminal, Fl-modified Lys excluded)

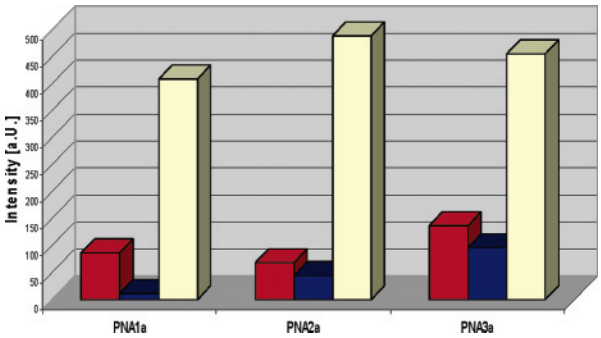


Figure 1. Fluorescence of 1 μM PNA **1a**−**3a** (red) (λ_{ex} = 572 nm, λ_{em} = 593 nm) in vitro is quenched upon addition of 1 equiv of Zn^{2+} (blue). Addition of 1 equiv of complementary DNA **4**−**6** opens the "molecular beacon", and fluorescence is strongly increased (yellow); pH 7 (1 mM MOPS), 100 mM NaCl. **DNA 4** 5′-TAGTTGTGA-3′, **DNA 5** 5′-AGTTGTGTA-3′, **DNA 6** 5′-ATGGTGAGCAAGGGCGAGGA-3′.

Uptake of PNAs **1**−**3** by HeLa cells was quantified by flow cytometry (Figure 2). Compared to **1b**−**3b**, the tpy modification in **1a**−**3a** increased cellular fluorescence 7.2−25.2-fold. Also, **1a** entered cells much more effectively than tetra-Lys-PNA **1c**, in particular, when taking into account the stronger fluorescence of the latter. A C-terminal tetra-Lys modification was reported to modestly improve cellular delivery of PNA and redirect gene splicing.[14] Remarkably, in case of the PNA 9mers **1a** and **2a** (but not of the 20mer probe **3a**), cellular fluorescence was 5.5 and 1.5-fold, respectively, higher in the presence of 1 equiv of Zn^{2+}, indicating a significant enhancement of PNA uptake by Zn^{2+}. This PNA sequence-dependent effect appears to be related to extracellular coordination of Zn^{2+} to the probe (Supporting Information, Figures

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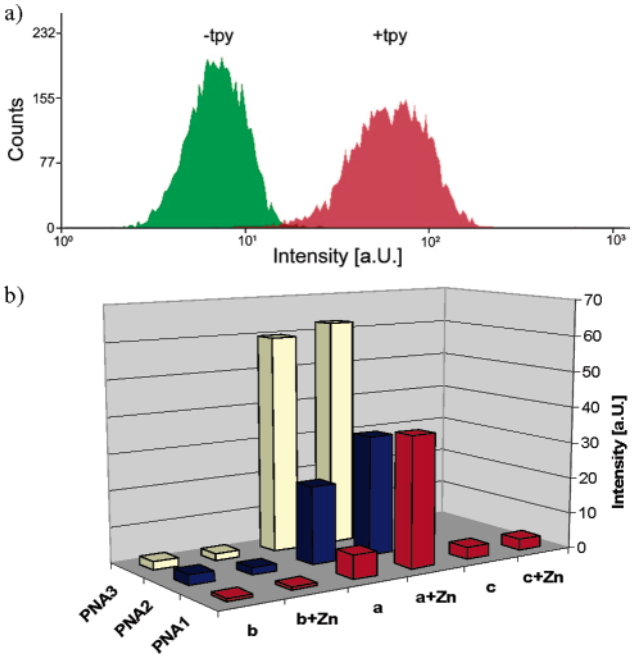

Figure 2. Flow cytometry analysis of HeLa cells incubated with 2.5 μM probe for 1 h. (a) Intensity counts for PNA **3b** + Zn^{2+} (green) and PNA **3a** + Zn^{2+} (red) (1 equiv of $ZnSO_4$). (b) Mean cellular fluorescence for various probes in the absence and presence of zinc ions, corrected for autofluorescence.

1s and 2s). It is unlikely in our view that such a complex, with the relatively weak Zn−chelator tpy (complex formation constant log$K$ = 6 for unmodified tpy),[15] remains intact in a zinc-sequestering[16] cytoplasmic environment. The tpy modification potentially enables selective cellular uptake of PNA by zinc-rich tissue (such as pancreas, testis, certain carcinoma, and the central nervous system) after stimulated zinc efflux from cells.[17]

Intracellular distribution of the probes in live HeLa cells was analyzed by confocal fluorescence microscopy, directly after incubation with 2 μM probe for 1 h (Figure 3). Typically, some fluorescence was observed in the cytoplasm, but nuclear staining was more pronounced. Both residual fluorescence (Figure 1) of the quenched probes and enhancement by nonspecific interactions (e.g., with proteins) might contribute to overall cellular fluorescence. Addition of Zn^{2+} did not seem to influence intracellular distribution of the probes. Analogues of **1a** in which the rhodamine fluorophore was replaced by coumarin (λ_{em} = 494 nm) and nile red (λ_{em} = 655 nm) also displayed efficient cellular and nuclear uptake (Supporting Information, Figure 3s). The lack of cellular import of **2a** at 4 °C may indicate an active cellular process.

In conclusion, the 2,2′:6′,2″-terpyridine (tpy) modification is readily introduced into PNA and efficiently promotes cellular and nuclear delivery. Zinc-enhanced cellular delivery is a first step toward a stimulus-controlled, tissue-selective uptake of PNA probes or reagents. Sequence-specific intracellular visualization of nucleic acid targets and the modulation of gene expression by tpy−PNA are under investigation.

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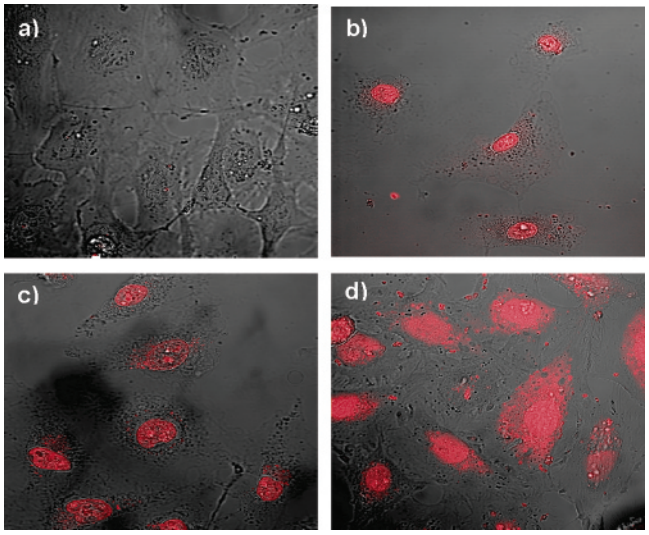

Figure 3. Confocal microscopy of living HeLa cells directly after incubation with 2 μM probe for 1 h (overlay DIC and rhodamine images). (a) PNA **2b** + Zn^{2+}; (b) PNA **2a** + Zn^{2+}; (c) PNA **2a**; (d) PNA **3a** + Zn^{2+} (1 equiv of $ZnSO_4$).

Supporting Information Available: PNA synthesis and characterization, T_m measurements of **1a**, flow cytometry (exp. details), UV titration of **1a** + Zn^{2+}, confocal microscopy of PNA **1a′-a″** intracellular distribution. This material is available free of charge via the Internet at http://pubs.acs.org.

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