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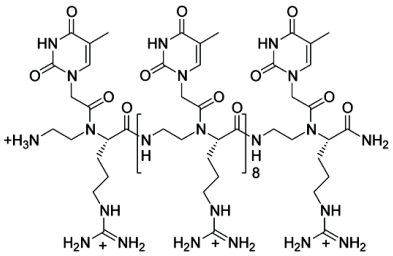

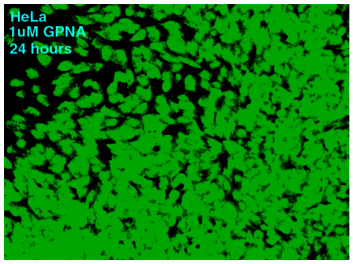

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Novel Binding and Efficient Cellular Uptake of Guanidine-Based Peptide Nucleic Acids (GPNA)

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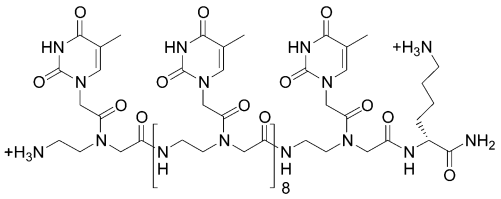
Peptide nucleic acid (PNA) is a synthetic analogue of DNA and RNA in which the natural sugar−phosphate backbone has been replaced by achiral *N*-(2-aminoethyl) glycine units (Scheme 1).[1] PNA can form sequence-specific hybrids with complementary DNA and RNA strands in accordance with the Watson−Crick base pairing rules. The resulting hybrid duplexes exhibit exceptional thermal stability, while the unnatural backbone renders PNA stable to both nucleases and proteases. Since its initial report, PNA has spawned considerable interest in its development as therapeutics, diagnostics, and molecular tools for basic research.[2]

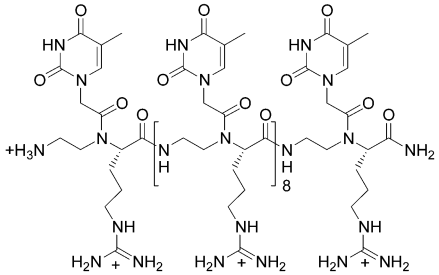
However, the promise of PNA in in vivo applications has dissipated with the discovery that PNA is not taken up readily by mammalian cells. Previous studies involving PNA have been performed in vitro,[3] with the exception of direct microinjection and,[4] more recently, through the use of carrier peptides.[5] This inherent limitation has prevented PNA from finding widespread applications in therapeutics as well as in basic research involving live cells and intact organisms. The growing awareness of these unfulfilled promises of PNA has prompted us to investigate a novel strategy for delivery of PNA into mammalian cells.

Our approach was inspired by the remarkable uptake properties of the human HIV-1 Tat transduction domain−which comprises relatively short basic sequence (GRKKRRQRRR).[6] Fusion of this domain to other proteins (regardless of size)[7] and synthetic molecules (including nanoparticles)[8] facilitated effective uptake of these complexes into mammalian cells. Intraperitoneal (IP) injection of these fused proteins into mice resulted in systemic uptake by cells in every tissue, including those in the brain regions.[9] More intriguingly, these fused proteins remained fully active in vivo.[10] Wender and colleagues have recently shown that TAT transduction domain could be replaced entirely with a homoarginine peptoid without compromising uptake efficiency.[11] On the basis of these findings, we hypothesized that incorporation of the arginine side chain (guanidinium functional group) into the PNA backbone (GPNA, Scheme 1) would effectively facilitate PNA uptake into mammalian cells, not only in cultures but also in intact mammalian organisms.

By starting with arginine instead of glycine in the synthesis of PNA backbone, we introduced the guanidinium functional group into the PNA backbone (Scheme S1, Supporting Information).[12] Thymine was initially chosen for this study because it presented the fewest synthetic challenges; however, it can be extended to other natural and unnatural nucleobases. The GPNA $(T_g)_{10}$ (PNA oligomer containing 10 thymines with arginine side chain installed at the α-L-position) was constructed from Boc-Arg(Tos)-T-OH monomers using Boc-protected solid-phase synthesis protocol, and the final oligomer was purified by HPLC and characterized by MALDI-TOF (*m*/*z* found 3673.2 and calcd 3675.0). The GPNA was found to be highly soluble in water compared to the unmodified PNA, which has a strong tendency to aggregate in solution.

Scheme 1





To determine whether GPNA was capable of recognizing and binding to a complementary DNA sequence (A_{10}), we measured the thermal stability of a GPNA−DNA mixture at 1:1 and 2:1 ratios utilizing UV spectroscopy. Our results showed that the mixture yielded a well-defined melting curve at both ratios with the T_m value of 78.0 °C (4.8 °C higher than that of the unmodified PNA_2−DNA triplex) (Figure S1, Supporting Information). Hybridization of GPNA to DNA containing internal T−C and T−T mismatches lowered the T_m by ∼10 °C, while a T−G mismatch destabilized the duplex by ∼5 °C (Figure S2, Supporting Information). These mismatched ΔT_m are comparable to those observed with unmodified PNA. On the other hand, GPNA containing a sequence identical to that of DNA did not yield defined melting transition. Taken together, these results suggest that, despite the positive charged side chains, GPNA retains high level of sequence specificity normally exhibited by PNA.

The binding properties of GPNA were further characterized by circular dichroism (CD). Figure 1 shows the CD spectra of single-stranded DNA and GPNA, GPNA−DNA duplex, and PNA_2−DNA triplex. The DNA duplex is characterized by a right-handed Cotton effects,[13] in contrast to the left-handed helix formed by PNA_2−DNA triplex as the result of the L-lysine residue at the C-terminus. GPNA by itself did not show CD signal, indicating the absence of secondary structures normally observed with unmodified PNA. However, in the presence of complimentary DNA, GPNA induced a left-handed split CD signal characteristic of a left-handed helix (Figure 1). A Job plot was further utilized to probe the binding stoichiometry between GPNA and DNA. It has been well estab-

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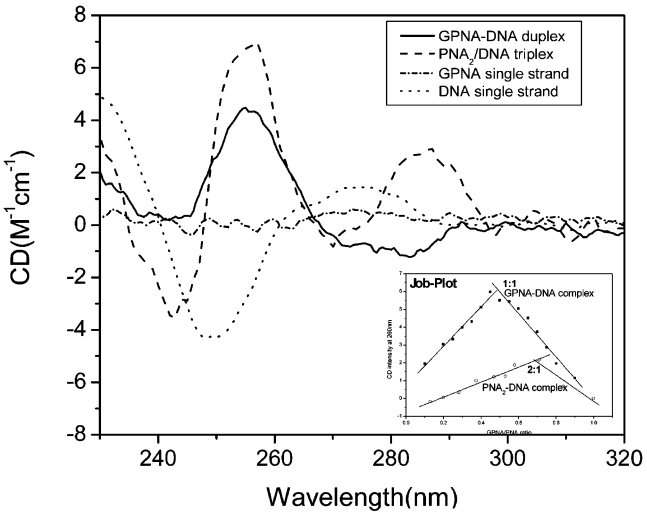

Figure 1. CD spectra of single-stranded DNA and GPNA, GPNA−DNA duplex, and PNA_2−DNA triplex. The hybridization complexes were formed by heating to 90 °C for 5min, followed by gradual cooling to room temperature. GPNA−DNA complex contained 2 μM of DNA and GPNA strand each, while that of PNA_2−DNA triplex contained 4 μM of PNA and 2 μM of DNA. Buffer contained 20 mM NaCl and 10 mM NaPi (pH 7.0). Inset: Job plots of GPNA−DNA and PNA_2−DNA complexes. The overall concentration was 2 μM, and the ratios between PNA and GPNA with DNA were varied. CD intensity was measured at 254 nm.

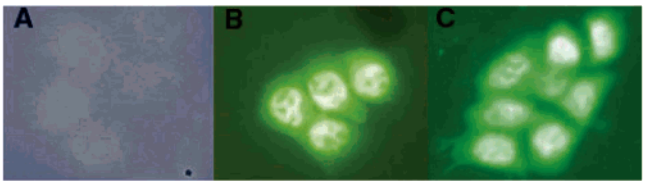

Figure 2. Fluorescence microscope images of HCT116 cells following incubation with unmodified PNA (A: Fl-T_{10}-Lys), TAT domain (B: Fl-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg), and GPNA (C: Fl-$(T_g)_{10}$), respectively. Cells were incubated with each respective oligomer at 37 °C for 10 min at 1 μM, followed by a thorough wash (5×) with PBS and fixing with 4% paraformaldehyde. Cells were then imaged with confocal fluorescence microscope.

lished that unmodified PNA T_{10} binds to its complementary DNA strand in a 2:1 ratio (PNA_2−DNA) to form a triplex. A similar experiment performed with GPNA, however, showed that the inflection point occurred at a 1:1 ratio, suggesting that polypyrimidine GPNA (in this case T_{10}) binds to complementary DNA to form a duplex rather than a triplex. Steric and electrostatic effects may play an important role in such binding preference.

Next, cellular uptake experiments were performed to examine the uptake efficiency of GPNA. GPNA, unmodified PNA, and TAT transduction domain (amino acids 49−57) were covalently linked to fluorescein at the N-terminus and their uptake properties were evaluated with human HCT116 (colon) and Sao-2 (osteosarcoma) cell lines using fluorescence microscope. Figure 2 shows the relative uptake efficiency of these oligomers by the HCT116 cell line. As shown in these images, GPNA traversed the cell membrane as effectively as the TAT transduction domain under identical conditions (Figure 2B and 2C). At the same time, the unmodified PNA did not show any uptake activity under the same condition (Figure 2A). More remarkably, TAT and GPNA appeared to localize specifically in the nucleus.

A more convincing evidence of this uptake selectivity came from 3D-rendering (data not shown) and superimposition of DIC (Differential Interference Contrast) and fluorescent images of individual cells treated with GPNA, which clearly showed that the fluorescence intensity was strongest in the nucleus (Figure S3, Supporting Information). Likewise, a parallel experiment utilizing DAPI (localizes specifically to the nucleus) as a second staining agent showed that the images acquired from the DAPI and FITC channels overlapped, again confirming that GPNA localized to the nucleus (Figure S4, Supporting Information). PNA containing alternating PNA units and arginine amino acids (NH_2-Arg-T-Arg-G-Arg-T-Arg-A-Arg-C-Arg-G-Arg-T-Arg-C-Arg-A) (Scheme S2, Supporting Information) also showed similar uptake properties. These results are in agreement with earlier reports for TAT and β-TAT transduction domain,[14] which indicated that the inter-distance between each arginine side chain along the backbone has little influence on uptake efficiency.

Although the mechanism of TAT transduction has not been fully substantiated, several groups have demonstrated that endocytosis does not play a significant role in this process since the rate of uptake is independent of temperature.[15] Likewise, we found that there was no difference in the uptake properties of GPNA at 37 and 4 °C, suggesting that the uptake mechanism of GPNA is neither endocytosis-driven nor receptor mediated (perhaps through membrane flipping).

In summary, we have demonstrated that PNA containing arginine side chains exhibited remarkable uptake properties while maintaining Watson−Crick recognition with complementary DNA strands. These findings provide tantalizing evidence suggesting that PNA could be selectively modified to facilitate uptake into mammalian cells. The ability to effectively introduce PNA into mammalian cells will be critically important for in vivo applications.

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Supporting Information Available: Synthetic scheme, UV melting curve, 3D-rendering, superimposed images of DAPI and fluorescein-labeled G-PNA, and schematic drawing of PNA oligomer containing alternating PNA units and arginines. This material is available free of charge via the Internet at http://pubs.acs.org.

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