Nucleic Acids

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Nucleic Acid with Guanidinium Modification Exhibits Efficient Cellular Uptake**

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Both chemical and biological technologies have been used in the development of oligonucleotides as therapeutic agents. Antigene and antisense oligonucleotides, aptamers, and

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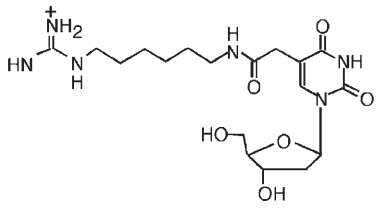
ribozymes (catalytic RNAs) are typically used for the regulation of gene expression.[1–4] The recent discovery of RNA interference promises an efficient gene regulation method for both basic and therapeutic research.[5–7] However, the cell membrane is a formidable barrier against the delivery of nucleic acids,[8–10] and therefore effective implementation of oligonucleotide technology depends on the efficient transfection of oligonucleotides. Thus, the enhancement of oligonucleotide uptake for targeted delivery is still a topic of great interest.

Cationic peptides are efficient tools for the transfection of oligonucleotides and cationic liposomes,[11,12] polycationic dendrimers,[13] and polyethyleneimine,[14,15] but if antisense oligonucleotide–cationic peptide conjugates are used in cellular delivery, the conjugates are often insoluble.[16] Furthermore, the conjugation is still not chemically straightforward and needs improvement,[17] and the peptide inhibits the function of the nucleic acid to which it is conjugated.[18] In the development of oligonucleotide technology, direct chemical modification of DNA or RNA is one method of improving function or adding a novel function, because the oligonucleotide-based structure and synthesis allow easy conjugation to DNA and RNA molecules for biochemical and biophysical applications. For example, a base-pair-mimic nucleoside induces site-selective RNA cleavage,[19] and the creation of modified DNA analogues with fluorescent aromatic compounds allows the rapid combinatorial screening and discovery of color-changing sensors of light exposure from a library.[20] Therefore, a modified nucleic acid analogue with efficient cellular uptake would be a useful tool for biochemical applications.

Herein, we report the initial synthesis of 5-[(6-guanidiniohexylcarbamoyl)methyl]-2′-deoxyuridine (**Tg**), which has a guanidinium group at the C5 position of deoxyuridine (Figure 1a). The guanidinium group is a side chain of arginine (Arg). Previous studies revealed that basic peptides such as

a)



b)

	T_m / °C
core duplex FAM-dTGTGAAGTGTCCCAGCCTGT dACACTTCACAGGGTCGGACA	71
core-T_4 duplex FAM-dTGTGAAGTGTCCCAGCCTGT $(T)_4$ dACACTTCACAGGGTCGGACA	72
core-$\mathbf{Tg}_4$ duplex FAM-dTGTGAAGTGTCCCAGCCTGT $(\mathbf{Tg})_4$ dACACTTCACAGGGTCGGACA	71

Figure 1. a) 5-[(6-Guanidiniohexylcarbamoyl)methyl]-2′-deoxyuridine (**Tg**); b) sequences and melting temperatures (T_m) of the core duplex, core-T_4 duplex, and core-$\mathbf{Tg}_4$ duplex (3 μM each) in a buffer containing NaCl at 100 mM. FAM = 6-carboxyfluorescein.

human immunodeficiency virus type 1 (HIV-1) Tat-(48-60) have membrane permeability and carrier function for intracellular protein delivery.[21] Moreover, many Arg-rich peptides such as HIV-1 Rev-(34-50) and octaarginine (Arg_8) can translocate efficiently through cell membranes.[22,23] Therefore, it is expected that **Tg** is likely to perform as an excellent transmembrane carrier for oligonucleotides. Herein, the guanidinium-modified DNA clearly shows cellular uptake in HeLa cells without any reagents.

A 20-mer DNA oligomer labeled with 6-carboxyfluorescein (FAM) at the 5′ end was prepared as the core strand: 5′-FAM-dTGTGAAGTGTCCCAGCCTGT. Then, four **Tg** moieties were added to the core at the 3′ end by a primer-extension reaction. After optimization of the reaction conditions (Supporting Information), the primer-extension reaction was performed with DNA polymerase. The primer extension used in this study might be useful for further applications because its products are easily purified. Next, the effect of the modified DNA (core-$\mathbf{Tg}_4$) on duplex stability was measured, because modified DNA base analogues often destabilize duplex formation. The melting temperatures (T_m) of the 20-mer core duplex (5′-FAM-dTGTGAAGTGTCCCAGCCTGT-3′/5′-ACAGGCTGGACACTTCACA-3′), a duplex in which the core strand has four dangling T nucleotides at the 3′ end (core-T_4 duplex: 5′-FAM-dTGTGAAGTGTCCCAGCCTGT$(T)_4$-3′/5′-ACAGGCTGGACACTTCACA-3′), and a core duplex with $\mathbf{Tg}_4$ at the 3′ end (core-$\mathbf{Tg}_4$ duplex: 5′-FAM-dTGTGAAGTGTCCCAGCCTGT$(\mathbf{Tg})_4$-3′/5′-ACAGGCTGGACACTTCACA-3′) were measured by UV/Vis spectroscopy in a buffer containing NaCl (100 mM), in close approximation to typical physiological conditions (Figure 1b). The T_m values of the core, core-T_4, and core-$\mathbf{Tg}_4$ duplexes (3 μM each) were 71, 72, and 71 °C, respectively. The data indicate that $\mathbf{Tg}_4$ as a dangling end does not noticeably inhibit duplex stability; these data are in agreement with previously observed trends. The 3′ dangling ends in B-form DNA are not energetically favored as a result of fewer geometric intrastrand stacking events.[24]

Next, the uptake of single-stranded core-$\mathbf{Tg}_4$ by HeLa cells was studied with both fluorescence-activated cell sorting (FACS) and microscopy. In typical experiments, HeLa cells were cultured in Eagle's minimum essential medium containing single-stranded core-$\mathbf{Tg}_4$ (4 μM) for 48 hours, washed with phosphate-buffered saline, and quantified by fluorescence measurement. The uptake of core-$\mathbf{Tg}_4$ was monitored by FACS analysis of FAM (Supporting Information). After 48 hours, cells incubated with core-$\mathbf{Tg}_4$ had higher fluorescence than a population of cells incubated in the absence of core-$\mathbf{Tg}_4$. To confirm the role of the guanidinium group, we synthesized 5-[(6-aminohexylcarbamoyl)methyl]-2′-deoxyuridine (**Ta**),[25] which has a Lys side chain (Supporting Information); it is known that Arg peptides undergo cellular uptake more readily than Lys peptides.[26,27] If the cellular uptake does not depend on the guanidinium group, then one would expect the cellular uptake efficiency of **Tg**- and **Ta**-modified DNA to be the same. Quantitation of FAM by FACS analysis showed that the uptake of core-$\mathbf{Ta}_4$ was 1.8% of that of core-$\mathbf{Tg}_4$, which suggests that the guanidinium group works efficiently as a carrier.

The microscopy images of HeLa cells cultured in the presence of 4 μM core-$\mathbf{Tg}_4$ are shown in Figure 2. Figure 2a shows a fluorescence overlay image of phase contrast, core-$\mathbf{Tg}_4$, and nuclei of living cells. The green and blue colors are a result of fluorescence of the FAM-labeled DNA oligomer and stained nuclei, respectively. As the cells incubated with the core (FAM-labeled DNA without $\mathbf{Tg}_4$) did not exhibit this green color (Supporting Information), the images clearly show that the chemical modification induces the cellular uptake activity. Moreover, the green areas do not seem to overlap the blue, which suggests that the cellular localization of these compounds is in the cytoplasm. Confocal microscopy studies were carried out to determine whether the guanidinium-modified DNA is internalized or simply attached to the cell surface. Fixed cells are usually used for confocal microscopy to obtain clear images, but recent studies indicate that cell fixation leads to the artifactual redistribution of cationic peptides into the nucleus.[28] Therefore, images of fixed cells and living cells were obtained (Figure 2b,c). Interestingly, these images showed that the distribution of the green color observed in the cytoplasm was similar to that observed with the fluorescence microscope. This suggests that the cellular localization of these compounds is mainly cytoplasmic. Recent studies on cationic peptides, such as the Tat peptide, support the notion that uptake occurs through an endocytosis pathway and that most cationic peptides reside in cytosol.[29–32] Our result for modified DNA is consistent with this behavior of cationic peptides. Although detailed future investigations are necessary, the intracellular redistribution of the modified DNA might occur through endocytosis.

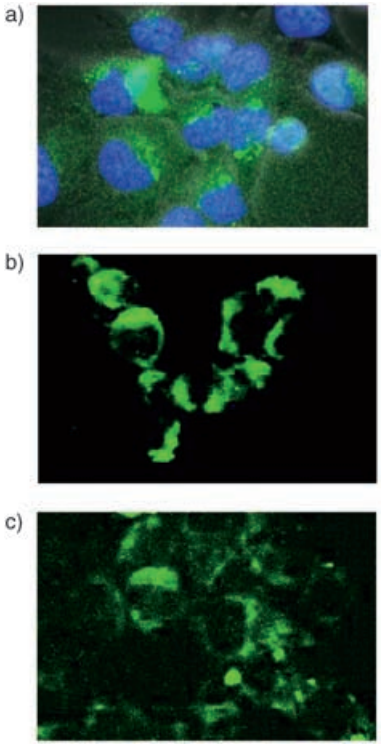


Figure 2. Microscopy images of HeLa cells cultured in the presence of core-$\mathbf{Tg}_4$ (4 μM): a) fluorescence overlay image of DNA oligomer, nuclei, and phase contrast of living cells; b) confocal microscopy image of DNA oligomer of fixed cells; c) confocal microscopy image of DNA oligomer of living cells.

Finally, the effect of modified DNA length on the cellular uptake efficiency was investigated (Figure 3). The amount of cellular uptake with core-$\mathbf{Tg}_3$ was 70% of that of core-$\mathbf{Tg}_4$. Interestingly, the amount of cellular uptake with core-$\mathbf{Tg}_6$ was only 30% of that of core-$\mathbf{Tg}_4$. Even at DNA concentrations that were twice as high (8 μM), the order of the amount of cellular uptake was still core-$\mathbf{Tg}_4$ > core-$\mathbf{Tg}_3$ > core-$\mathbf{Tg}_6$. In the case of Arg peptides, the efficiency of cellular uptake decreased with increasing polypeptide length because of aggregation. The length range showing more efficient uptake was between eight and ten Arg residues.[23] Our data are in agreement with an increase in length being unfavorable for cellular uptake. However, the most favorable length (four **Tg** nucleotides) is shorter than an eight- to ten-residue poly-Arg peptide. Although the reaction conditions of core-$\mathbf{Tg}_4$ were not optimized, the degree of cellular uptake for core-$\mathbf{Tg}_4$ at 8 μM with incubation for 48 hours was about 75% that of the uptake for the core through a commercial liposome transfection agent (LipofectAMINE 2000 reagent (Invitrogen)) under optimized conditions. This indicates that the cellular uptake of core-$\mathbf{Tg}_4$ is not so inefficient.

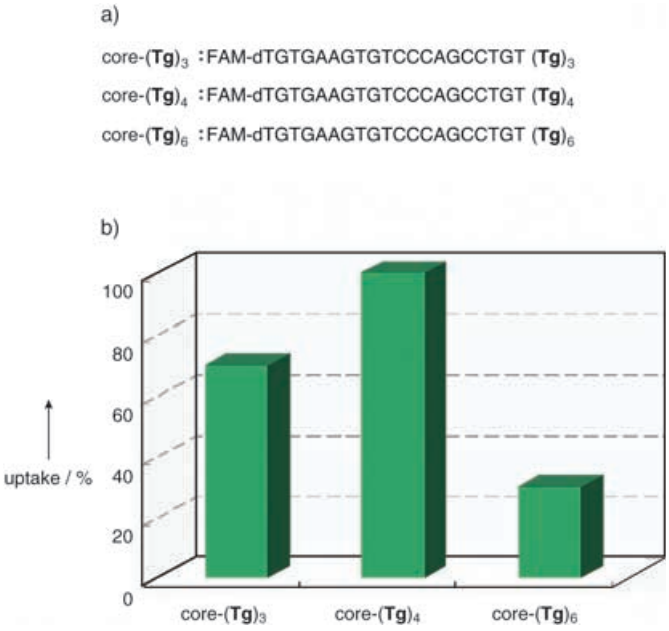


Figure 3. a) DNA sequence studied to investigate the effect of modified DNA length on cellular uptake. b) Uptake efficiency relative to core-$\mathbf{Tg}_4$ quantified by FACS analysis of FAM.

In summary, we have found that 5-[(6-guanidiniohexylcarbamoyl)methyl]-2′-deoxyuridine (**Tg**) plays the important role of carrier for cellular uptake. In the case of the Arg-rich peptide, HeLa cells are a typical example of a cell type that is difficult for intracellular peptide delivery.[23] Therefore, the cellular uptake of **Tg** in HeLa cells indicates that **Tg** could be applied as a carrier agent for a wide range of cell types. The activity of **Tg** as the carrier was observed not only for HeLa

cells but also for RAW 264.7 cells, which are known to allow uptake readily (Supporting Information).[23] Interestingly, the optimal number of **Tg**-modified bases as a carrier for a 20-nucleotide DNA oligomer was only four. Oligonucleotide-based functional molecules allow easy conjugation to DNA and RNA for biochemical and biophysical applications. Thus, **Tg** could be used as a new tool in future applications for intracellular oligonucleotide delivery.

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