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A Peptide Nucleic Acid Embedding a Pseudopeptide Nuclear Localization Sequence in the Backbone Behaves as a Peptide Mimic

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In this paper, the synthesis of a short modified peptide nucleic acid (PNA), obtained by using several different L-amino acids as synthons, is shown. The synthesis was performed by a submonomeric strategy, obtaining a model trimeric PNA containing embedded amino acid derived side chains in its backbone that mimic the peptide sequence PKKKRKV, which is a nuclear localization signal (NLS) widely used for translocating cargo molecules into cell nuclei. Fluorescence experiments demonstrated that this modified PNA, and not a standard unmodified PNA having the same nucleobase sequence, was able to penetrate Rhabdomyosarcoma cell nuclei, exactly behaving as the NLS standard peptide.

Introduction

Peptide nucleic acids (PNAs, Figure 1) are DNA analogues, first introduced by Nielsen and co-workers in 1991,[1] which demonstrate superior ability in specific DNA binding through standard Watson–Crick hydrogen bonds. PNAs are able to specifically bind DNA and RNA to give PNA–DNA and PNA–RNA duplexes that are usually more stable than DNA–DNA and DNA–RNA duplexes.[2] A particularly fascinating, but seldom explored, aspect of PNAs is their "double nature": PNAs are not only able to recognize a complementary DNA sequence through the nucleobases (a property typical of nucleic acids), but they also possess a pseudopeptidic backbone and therefore might potentially display properties similar to those performed by proteins. Indeed, almost all the papers published on PNAs have so far explored their "nucleic acid" characteristics (recognition of complementary sequences) and almost no one has concentrated on their "peptide" characteristics. The reason for this asymmetry is easily understood by considering the structure of standard PNAs: the backbone can be considered as a long sequence of pseudoglycylglycine dipeptides; therefore, a mimic of a polyGly protein. In order to express all the "proteic potential" of PNAs, the backbone should be modified to become a real peptide mimic, by inserting amino acid derived side chains in the 2- and 5-positions (Figure 1). In this way, a real "peptide" and "nucleic acid" could be obtained, fully exploiting the intrinsic double nature of the molecule.

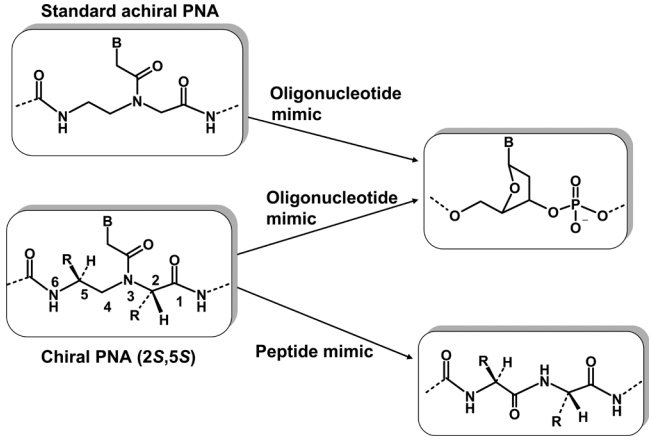

Figure 1. A standard PNA compared with a 2*S*,5*S*-modified PNA: both are able to perform DNA recognition, but the backbone of the latter also has the potential to mimic a peptide sequence.

The first steps toward the exploration of the full potential of PNAs as "peptide mimics" have been taken by our group in the last years by synthesizing 2- and 5-substituted chiral PNAs.[3] Seminal studies have been devoted to establishing the basic rules by which the configurations of the single stereogenic centers, independently from the type of side chains, influence the overall PNA conformation and in turn how this conformation affects the DNA binding ability, both in terms of specificity and affinity.[4] In particular, it has been found that the insertion of a stereogenic center

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influences the preference of the PNA strand to adopt a right-handed or a left-handed helix, and as a general rule, PNAs preferring the right-handed helix will bind to DNA (which is right-handed) more effectively. The correct configurations for having right-handed PNAs in 2,5-disubstituted PNAs have been found to be 2*R*,5*S* and 2*S*,5*S*. Thus, a backbone including L-amino acid derived side chains at the 2- and 5-positions (Figure 1) has the potential to be a good mimic of natural peptides, formed by all L-amino acids, and at the same time preserving the correct handedness for DNA binding.

Although the introduction of substituents in the PNA backbone has been used to introduce functional groups and charges (thus modulating the properties of the PNAs),[5–7] the use of side chains mimicking an entire complex peptide consensus sequence with a specific biological function has never been reported, and this represents a challenging task both conceptually and synthetically.

In this paper we explore the ability of PNAs to act as a peptide mimic, reporting the design and the synthesis of a modified PNA containing an embedded mimic of a nuclear localization signal (NLS) in its backbone. NLS are short, predominantly basic, peptide sequences identified as nuclear import signals, which are translocated across the nuclear envelope by interaction with specific carrier proteins.[8] The sequence chosen to be embedded into the PNA backbone was PKKKRKV, a hydrophilic sequence derived from the simian virus 40 large T-antigen. This peptide has already been used, covalently linked to the molecule of interest, to carry antigen oligonucleotides[9] and antigen PNAs[10–12] in different cell nuclei. The modified PNA was synthesized and tested for its ability to enter into cell nuclei and then assayed by fluorescence microscopy. The modified PNA, but not a homologous standard achiral PNA, exactly behaved as the NLS peptide, that is, it entered inside the nuclei of RH30 cancer cells.

Results and Discussion

The structure of the NLS peptide (**1**) chosen to be embedded into the PNA backbone is represented in Figure 2. A model PNA consisting of only three residues was chosen as the substrate into which the peptidic structure would be embedded (**2**, Figure 2). The fusion of the two structures led us to design modified PNA **3** (Figure 2) containing embedded side chains derived from natural L-amino acids. The proline residue was linked as an amino acid residue at the N-terminus, for it bears a secondary amine and could not be included in a PNA structure. All compounds were synthesized with a rhodamine linked at the N-terminus, with an aminoethoxyethoxyacetyl (AEEA) spacer in between, so that it could be visualized in the interaction and cell penetration studies, and with a glycine residue at the C-terminus, for easiness of synthesis on the solid phase.

The syntheses of NLS peptide **1** and standard PNA trimer **2** were performed on solid phase by following standard Boc protocols. After cleavage from the resins, NLS peptide

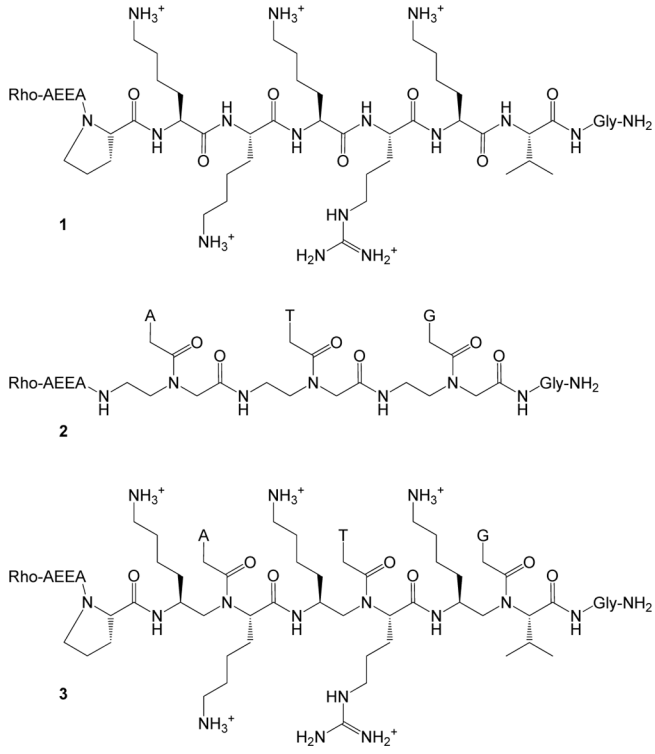
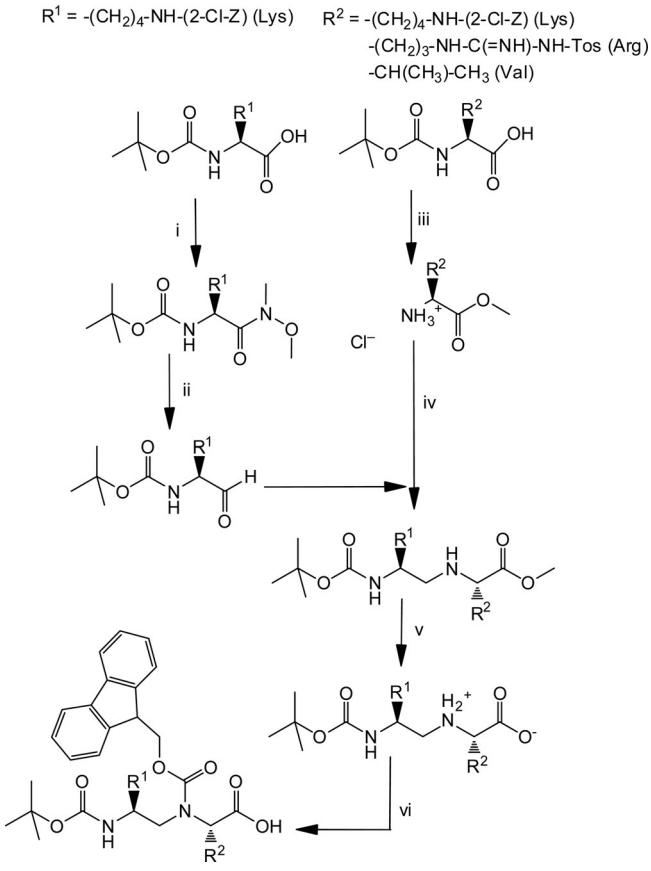


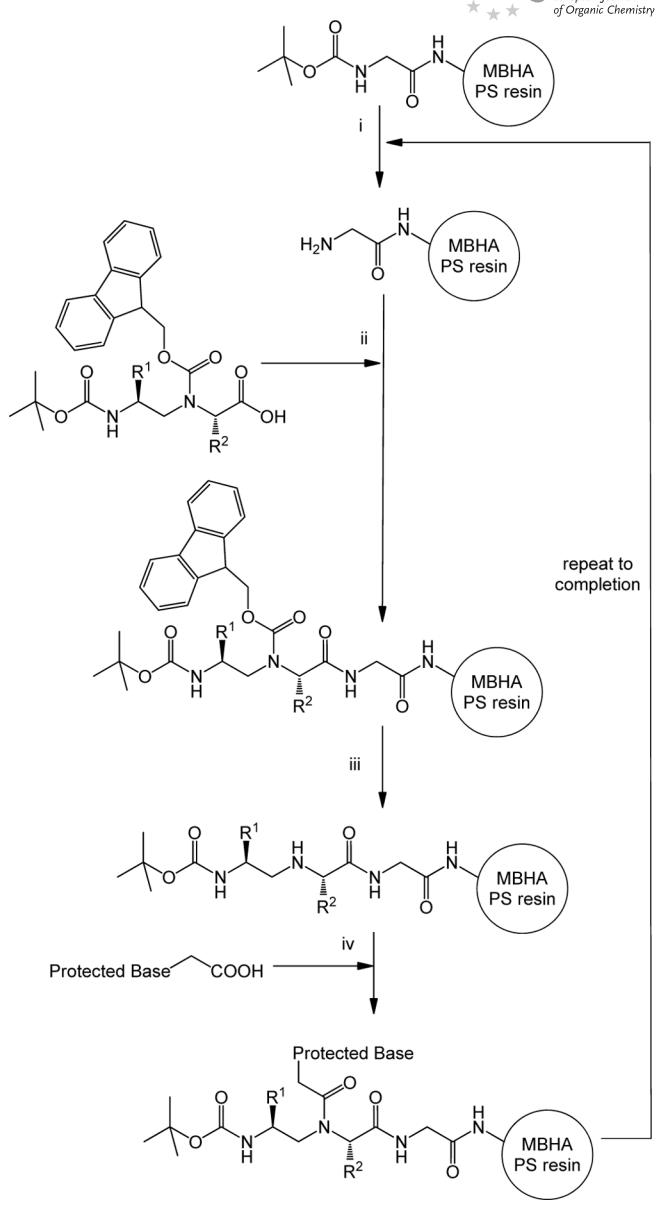
Figure 2. The NLS peptide PKKKRKV (**1**), a standard PNA trimer ATG (**2**), the modified PNA trimer ATG containing the embedded NLS sequence (**3**). A = adenine, T = thymine, G = guanine, Gly = glycine, Rho = rhodamine, AEEA = 2-aminoethoxyethoxyacetyl spacer.

1 and PNA trimer **2** were purified by RP-HPLC and characterized by ESI mass spectrometry (see the Supporting Information). A Boc submonomer strategy was used for modified PNA **3** to better preserve the optical purity at the 2-position.[13] The submonomers were prepared, as reported in Scheme 1, starting from the corresponding commercial Boc-L-amino acids suitably protected on the side chains. Boc-L-Lys-(2-Cl-Z)-OH, to be used as a synthon for inserting the lysine side chains into the 5-position, was transformed into the corresponding Weinreb amide and then reduced to the aldehyde. Boc-L-Lys-(2-Cl-Z)-OH, Boc-L-Arg-(Tos)-OH, and Boc-L-Val were transformed into the corresponding methyl esters and Boc deprotected by using HCl/MeOH. The PNA backbones were then synthesized by reductive amination of the L-amino acid methyl esters with the Boc-L-Lys-(2-Cl-Z) aldehyde. The methyl esters were hydrolyzed and the secondary amines were protected by the introduction of a Fmoc group (Scheme 1, details in the Supporting Information). All compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy and ESI mass spectrometry.

According to previously reported procedures,[13] the chiral submonomeric units were inserted by manual coupling with the HATU/DIPEA [*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate/*N*,*N*-diisopropylethylamine] protocol on a MBHA-PS (4-methylbenzhydrylamine polystyrene) resin, and after Fmoc depro-



Scheme 1. Synthesis of the submonomers. Reagents and conditions: (i) *N*-methyl-*N*-methoxyamine·HCl (0.97 equiv.), HBTU [*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate], DIPEA, DMF, η: 90%; (ii) $LiAlH_4$ (1 M in THF, 4.6 equiv.), THF, η: 75%; (iii) $SOCl_2$ (1 M in CH_3OH), η: quantitative; (iv) L–AA–OMe (1 equiv.), Boc-Lys-H (1 equiv.), DIPEA (1 equiv.), $NaBH_3CN$ (3 equiv.), CH_3COOH (1.1 equiv.), CH_3OH, η: 30–35%; (v) NaOH (10 equiv.), THF/H_2O = 1:1, then HCl up to pH 5.5, η: 87–90%; (vi) BTSA [*N*,*O*-bis(trimethylsilyl)acetamide] (2 equiv.), DIPEA (1.5 equiv.), FmocCl (2 equiv.), CH_2Cl_2, η: 30–35%.



Scheme 2. Solid-phase synthesis of the modified PNA containing the embedded NLS sequence by the Boc submonomeric strategy. Reagents and conditions: (i) neat TFA (added of 5% *m*-cresol); (ii) HATU/DIEA; (iii) piperidine/NMP 20%; (iv) carboxymethyl-Z-adenine, carboxymethyl-*O*-benzylguanine, or carboxymethylthymine, DIC/DhBtOH.

tection, the nucleobases residues were introduced by a double coupling with DIC/DhBTOH (*N*,*N*′-diisopropylcarbodiimide/3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; Scheme 2). After cleavage from the resin, the PNA trimer was purified by RP-HPLC and characterized by ESI mass spectrometry (see Supporting Information).

The ability of the different compounds to be internalized in the cell nuclei was assessed in vivo: RH30 cells were incubated with 10 μmol/L of peptide **1**, standard PNA **2**, or modified PNA **3** and analyzed after 3 h. Fluorescence microscopy images showed that after 3 h in the RH30 cell line, high intracellular levels with a main nuclear localization could be observed for NLS peptide **1** (Figure 3), as expected, and for modified PNA **3** (Figure 4), whereas standard PNA **2** was not detected inside the nuclei (Figure 5), confirming the previous results.[12,14]

The lack of nuclear fluorescence when standard PNA **2** was used demonstrates that these probes are not recognized by any carrier protein, and alternative pathways for cell penetration are absent, or inefficient under the conditions used for the experiment. NLS peptide **1**, on the other side, shows an uptake consistent with a receptor-mediated process, as already known.[9–12] Likewise, the concentration of PNA **3** in cell nuclei is a strong indication that this molecule is able to follow the same pathway as the NLS peptide. As a matter of fact, the uptake in the nuclear region and the homogeneous diffusion is indeed consistent with a receptor-mediated internalization process. Because the importin-mediated nuclear transport mechanism has been demonstrated for NLS peptide **1**, it can be assumed that modified PNA **3** may be able to interact with this receptor protein under the conditions used.

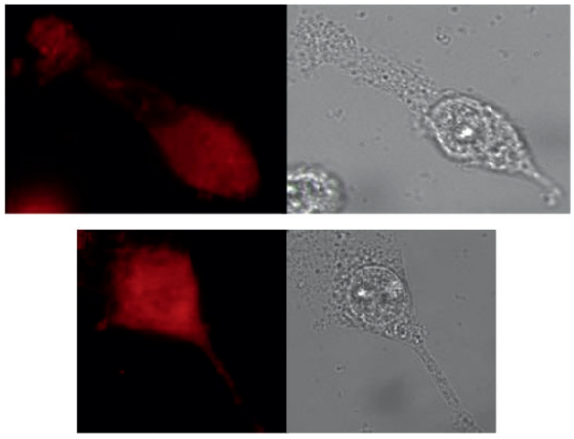


Figure 3. Fluorescence microscopy analysis (left) of uptake of RH30 cells treated for 3 h with NLS peptide **1**. Optical images are also reported (right).

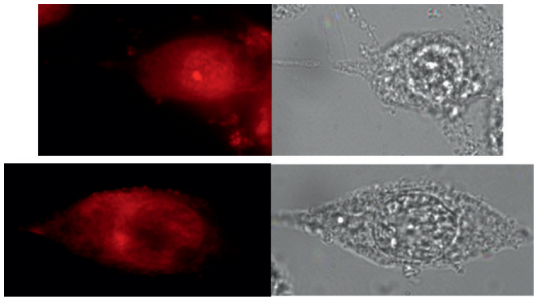


Figure 4. Fluorescence microscopy analysis (left) of uptake of RH30 cells treated for 3 h with modified PNA **3**. Optical images are also reported (right).

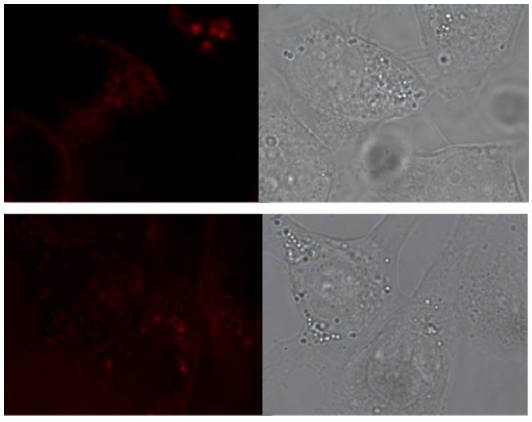


Figure 5. Fluorescence microscopy analysis (left) of uptake of RH30 cells treated for 3 h with unmodified PNA **2**. Optical images are also reported (right).

Conclusions

In conclusion, we have demonstrated the chemical feasibility of synthesizing peptide nucleic acids containing embedded amino acid side chains in their backbone that mimic a complex peptide sequence. The preliminary data here on the NLS-mimicking PNA demonstrate that the pseudopeptide sequence encrypted in the backbone of the modified PNA can behave as a fully functional peptide mimic, shedding light on a new dimension concerning the properties of PNAs, which may find numerous applications in bioorganic chemistry and molecular biology. For example, a PNA with the embedded NLS sequence could be used to efficiently translocate the PNA in the cell nuclei after injection in the blood stream, avoiding degradation from serum proteases, as it may happen when the NLS sequence is simply linked as a normal peptide at the C-terminus. In more general terms, a peptide nucleic acid possessing the functionalities of the proteins might be potentially used to develop PNAs endowed with catalytic functions, fully exploiting their intrinsic nucleic acid and peptide natures. More experiments are needed to obtain long PNAs enclosing these modified monomers at one terminus to verify their binding properties towards complementary nucleic acids.

Supporting Information (see footnote on the first page of this article): Experimental details, LC/ESI-MS and LC/UV profiles of compounds **1**–**3**.

Acknowledgments

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