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Stepwise Solid-Phase Synthesis of Oligonucleotide-Peptide Hybrids[1]

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Abstract: Oligonucleotide-peptide hybrids containing a nuclear transport signal have been synthesized stepwise on a polyethyleneglycol-polystyrene support using base labile protecting groups for the nucleobases and aminoacid side chains.

Modified and unmodified oligonucleotides have been widely employed to inhibit gene expression. In order to enhance the activity of oligonucleotides and their analogues, they have been covalently linked to intercalating, alkylating, photocrosslinking and radical generating reagents. Besides increasing the affinity for the target sequence, some of these compounds promoted the uptake of oligonucleotides by cells and improved their resistance to nucleases.[3-5] Covalent attachment of poly(L-lysine) and lipid molecules such as cholesterol to oligonucleotides enhances the antisense activity of these compounds.[6,7] In a previous report we have shown that oligonucleotide-peptide hybrids carrying a lysine-rich nuclear transport sequence form a more stable double helix structure than the unmodified oligonucleotide.[8] In the present communication we would like to describe a solid-phase protocol for the preparation of these hybrid molecules that uses the same solid support for the preparation of both peptide and oligonucleotide moieties.

The preparation of oligonucleotide-peptide hybrids in the same support has been described using the Fmoc group for the protection of the α-amino function and ^{t}Bu groups for the protection of side chains.[9-11] In our opinion, the described methods are not optimized for the preparation of the large quantities needed for antisense studies. The use of strong acids (trifluoroacetic acid) needed for the removal of ^{t}Bu groups will cause partial depurination of DNA. To avoid acid treatment when DNA is present we have designed the strategy shown in figure 1. Basically, the peptide is assembled first using the base labile (*o*-nitrophenyl)ethyl ester (NPE) linker,[12] the acid labile ^{t}Boc group as α-amino protecting group and the base labile Fmoc and Fm groups for the protection of lysine and aspartic acid lateral chains.[13] A linker molecule was added to connect the peptide part to the oligonucleotide by converting the last amino group of the peptide to an hydroxy group protected with a DMT group.[9-10] Afterwards, the oligonucleotide is assembled using the standard (Bzl, ibu) 2'-

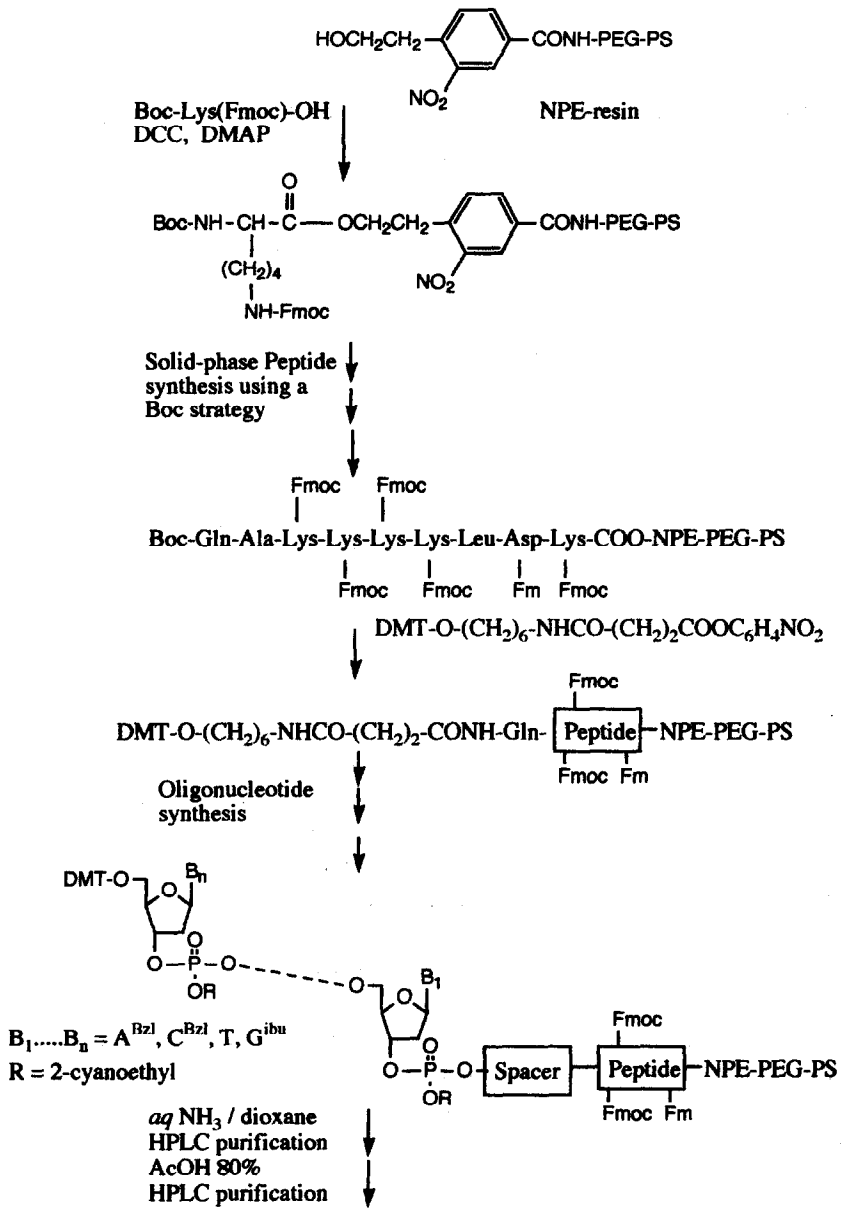

Figure 1 : Stepwise preparation of oligonucleotide-peptide hybrids.

deoxyribonucleoside 3'-O-(2-cyanoethyl) phosphoramidites. At the end of the synthesis, all protecting groups (peptide and oligonucleotide) and the hybrid-solid support linkage can be removed by treatment with concentrated aqueous ammonia.

We first tried the preparation of the peptide-oligonucleotide hybrids using controlled pore glass (CPG) as solid support. Unfourtunately, the assembly of the peptide[14] was not efficient enough generating a mixture of peptides with different lengths. Afterwards we tried polyethylene glycol-polystyrene (PEG-PS) because this support is used in solid-phase peptide synthesis[15] and it has been described recently for the preparation of oligonucleotides.[16] The nuclear transport consensus sequence found in Nucleoplasmin[17] Gln-Ala-Lys-Lys-Lys-Lys-Leu-Asp-Lys was assembled efficiently on NPE-polyethylene glycol-polystyrene solid support using Boc-amino acids bearing Fm and Fmoc protecting groups for the side-chains following a conventional protocol of synthesis used in our laboratories.[14] After deprotection of the Boc group, the linker[9-10] activated as its *p*-nitrophenyl ester was coupled in DMF for 2 h at 25 °C. The synthesis of the oligonucleotide was studied on a *home-made* manual synthesizer using DMT-thymidine 2-cyanoethyl phosphoramidite. We found that the addition of the nucleotides was strongly affected by the solvent used for the coupling reaction. When acetonitrile was used as solvent only 50% coupling yields were obtained. Using a 1:1 mixture of pyridine or DMF in acetonitrile gave 50% and 10% coupling yields respectively. Only good yields (>90%) were obtained when the phosphoramidite was dissolved in dry dichloromethane (0.1 M) and the tetrazole was dissolved either in dry acetonitrile or in dry tetrahydrofuran (0.4 M). Based on these observations, we studied the assembly of oligonucleotides on the peptide linked to the solid support using an automated DNA synthesizer. Standard (A^{Bzl}, C^{Bzl}, G^{ibu}, T) nucleoside 3'-O-(2-cyanoethyl) phosphoramidites were dissolved in dry dichloromethane (0.1 M) and installed in the DNA synthesizer. 1 μmol synthesis cycles were used as recommended for the supplier except for the coupling, capping, oxidation and detritylation times that have slighty increased. The DNA sequences prepared were : I) 5' TTTTT-peptide ; II) 5' AGC CCA GCT CAG CTC-peptide and III) 5'-Fluorescein-AGC CCA GCT CAG CTC-peptide. We used Fluorescein-ON phosphoramidite (Clontech, USA) for the incorporation of fluorescein at the 5'-end. Coupling yields were higher than 95% except for the coupling of fluorescein-ON phosphoramidite that was only 50%. At the end of the synthesis, the oligonucleotide-peptide-solid supports were treated with conc. aqueous ammonia (1 ml) - dioxane (0.1 ml) overnight at 50 °C. Oligonucleotide-peptide hybrids were purified using the standard two-steps HPLC purification. A first step to separate the truncated sequences from the DMT-containing product and a second HPLC after removal of the DMT with 80% acetic acid. Oligonucleotide-peptide hybrids were characterized by amino acid analysis after 6N HCl hydrolysis and by nucleoside analysis after snake venom phosphodiesterase and alkaline phosphatase digestion. Homogeneity was checked by 20% polyacrylamide-7 M urea gel electrophoresis. As it can be seen in figure 2, oligonucleotide-peptide hybrids have a lower mobility due to the presence of positive charges from lysines and the increased molecular weight of the hybrids.

In conclusion, we have shown that defined oligonucleotide-peptide hybrids containing peptides at the 3'-end of the oligonucleotide can be synthesized efficiently on the same solid support using a strategy of protecting groups, where base labile groups are used for the protection of both side- chain of amino acids and nucleobases. The method described here can be easily scaled-up for the preparation of large amounts of oligonucleotide-peptide hybrids needed for antisense studies. The study of the biological properties of these oligonucleotide-peptide hybrids is currently in progress.

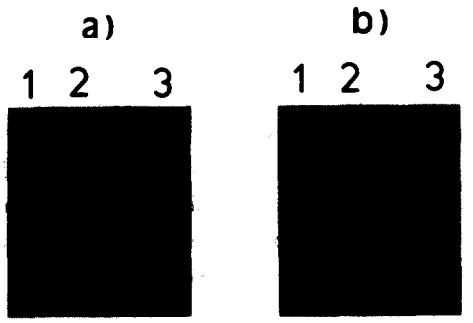

Figure 2: Analysis of oligonucleotide-peptide hybrids prepared in this work by 20% polyacrylamide-7M urea gel electrophoresis. a) Ethidium bromide stained gel observed under 302 nm light and photographed with a orange-red gelatine filter. b) the same gel analyzed at 302 nm without ethidium bromide staining and photographed with a yellow gelatine filter. 1) Fluorescein-labelled 15 mer having the same sequence as III but without peptide. 2) Oligonucleotide-peptide sequence II. 3) Fluorescein-labelled oligonucleotide-peptide sequence III.

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1. Abbreviations used are as follows: Boc: *tert*-butyloxycarbonyl; ^{t}Bu : *tert*-butyl; Bzl : benzoyl; CPG : controlled pore glass; DCC: dicyclohexylcarbodiimide; DIEA: *N,N*-diisopropylethylamine; DMAP: *N,N*-dimethyl- aminopyridine; DMF: *N,N*-dimethylformamide; DMT: dimethoxytrityl; Fm: 9-fluorenylmethyl; Fmoc: 9-fluorenylmethyloxycarbonyl; ibu: isobutyryl; HOBt: 1-hydroxybenzotriazole; NPE : (*o*-nitrophenyl)ethyl linkage; PEG : Polyethylene glycol spacer; PS: copoly(styrene-1%-divinylbenzene) polymeric support; TFA: trifluoroacetic acid.
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