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# Synthesis of Oligonucleotide-Peptide Conjugates Containing a KDEL Signal Sequence

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**Abstract:** An improved method of preparation of oligonucleotide-peptide conjugates is described. An oligopeptide containing $\alpha$ and $\epsilon$-aminogroups is mainly substituted at its $\alpha$-$NH_2$ end by $\epsilon$-maleimidocaproic acid-*N*-hydroxysuccinimide ester at pH 6.5 for 1 h. The $N_\alpha$-maleimidocaproyl-peptide derivative, purified by HPLC, reacts with the thiol group of an oligonucleotide in a 82% yield at pH 7.2. The thiol group is generated *in situ* by the action of tris(carboxyethyl)phosphine on an oligonucleotide bearing a disulfide bridge.

Synthetic antisense oligonucleotides suitable to control expression of viral genes[1] or oncogenes[2] are putative therapeutic agents. In order to enhance the activity of oligonucleotides or oligonucleotide analogues, these compounds have been covalently linked to peptides with defined functions. Examples include the use of a nuclear transport signal[3], a hydrophobic peptide[4] or polylysine[5].

It is known that newly synthetized proteins with a Lys-Asp-Glu-Leu (KDEL) sequence at their C-terminal end are retained in the endoplasmic reticulum[6]. Furthermore, it is in the endoplasmic reticulum that cytosolic peptides are processed to be presented by the MHC I system. On these bases, we prepared oligonucleotide-peptide conjugates made of an antisense oligodeoxynucleotide linked to a peptide with a KDEL sequence at its C-terminal end, with the aim of helping these conjugates to reach the endoplasmic reticulum and from there to cross the membrane and to enter the cytosol or/and the nucleus where the antisense oligonucleotide targets are located.

In this paper, we describe an improved method which allows to synthetize an oligonucleotide-oligopeptide conjugate by linking an oligonucleotide to a peptide selectively activated at its $\alpha$-amino group. The oligonucleotide containing a free thiol group (SH) at its 3'-end is reacted with a $N_\alpha$-maleimidocaproyl-substituted peptide. The oligonucleotide used is a 12-mer with a sequence $^{5'}$ACACCGACGGCG$^{3'}$ specific for Ha-*ras* around the point mutation in the 12$^{th}$ codon.

The synthesis of oligonucleotide-peptide conjugates, directly on solid-phase peptide or DNA synthetizers, has been reported[4,7], but this method is not satisfactory due to the lack of "universal" protecting groups for both strategies and to side reactions during coupling cycles or cleavage from the solid support. A postsynthesis conjugation appears to be a better alternative.

The conjugation through disulfide linkage of an oligonucleotide to a separately synthetized peptide has been reported[3],

but the S-S bridge will be reduced in endosomes[8] and therefore is not appropriate for the assigned purpose. For these reasons, we prepared oligonucleotides and peptides separately each one being adequately functionalized to obtain a conjugate through a thioether bond. Similar conjugations using the reaction of a maleimide group with a thiol group have been previously reported[9], but the maleimide group was appended at pH 8.0[10]. Gregory[11] showed that the maleimide group is stable between pH 5.5 to 7.0, but is readily hydrolyzed at pH 8.0 during the coupling step to give maleamic acid. In our procedure the maleimide group is appended to the $\alpha$-$NH_2$ of the peptide, at neutral pH, to preserve the $\epsilon$-$NH_2$ of lysine side chain which is essential for expression of the peptide biological activity. The synthesis strategy is outlined in Figure 1.

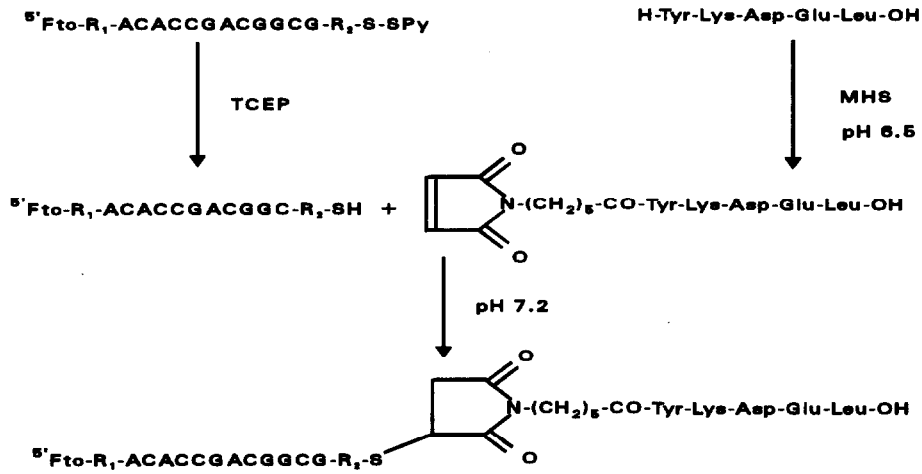


**Figure 1**: Synthesis scheme of oligonucleotide-peptide conjugate.
TCEP: [tris(2-carboxyethyl)phosphine], MHS: $\epsilon$-maleimidocaproic acid *N*-hydroxysuccinimide ester,
Ftc: fluoresceinthiocarbamyl, Py: 2-pyridinyl, $R_1$: $NH\text{-}(CH_2)_6\text{-}NH\text{-}CO$, $R_2$: $O\text{-}(CH_2\text{-}CH_2\text{-}O)_2\text{-}CH_2\text{-}CH_2$.

The peptide H-Tyr-Lys-Asp-Glu-Leu-OH (YKDEL) bearing a tyrosine residue for further radiolabeling of the conjugate was synthetized in solution using commercially available protected amino acids: Boc-Tyr(Bzl)-OH, Boc-Lys(Z)-OH, Boc-Asp(OBzl)-OH, Boc-Glu(OBzl)-OH and HCl,H-Leu-OBzl. Peptide coupling was performed by benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) mediated reaction in dichloromethane (DCM) (30 min); 40% trifluoroacetic acid in DCM was used for the Boc deprotecting step. After synthesis, the protecting groups were removed by catalytic hydrogenolysis. The peptide was analyzed and purified by reversed-phase chromatography using a C18 250 x 10 mm column (Merck) using a linear gradient from solvent A (water + 0.1% ammonium acetate) to solvent B (acetonitrile + 0.1% ammonium acetate). The gradient was run from 5% to 90% B for 25 min. The peptide is eluted at 14.0 min. The amino acid analysis was : Tyr(0.94), Lys(1.19), Asp(1.11), Glu(0.91) and Leu(1.00).

The oligonucleotide bearing a 3'-disulfide bridge and a 5'-$NH_2$ group was synthetized by solid phase synthesis (Applied Biosystems) on a disulfide derivatized solid support (10 $\mu$mole scale)[12] using the phosphoroamidite method and the fast oligonucleotide deprotection phosphoroamidite base protecting groups (FODs™). The detritylated 5'-end of the protected oligonucleotide (uncleaved from the support) was activated for 5h at 20°C with 0.3M 1,1'-carbonyldiimidazole in 5ml of dioxane and then treated for 12h with 0.45M hexamethylene diamine in 5mL of pyridine. After cleavage from the solid phase and deprotection with concentrated ammonia at 55°C for 1 h, the oligomer 3'-disulfide was substituted on

its 5'-aminohexylaminocarbonyl end by introducing a fluorescein group required to study the intracellular traffic of the conjugate. The disulfide bridge of the fluorescent oligonucleotide was reduced with tris(2-carboxyethyl) phosphine TCEP[13] to give the 12 mer 3'-thiol which was converted into the 3'- dithiopyridinyl derivative (SS-Py) by reaction with 2,2'-dithiodipyridine at pH 7.0. Oligonucleotides were purified by ion-exchange chromatography on a DEAE 8HR 100 x 10mm column (Waters) using two buffers: A [25 mM Tris HCl, 1mM EDTA, pH 8.0 (90%)/acetonitrile (10%)] and B (buffer A +1.0 M NaCl). A linear gradient was run from 10% to 80% B in 40 minutes. The oligonucleotide substituted with Ftc and SS-Py at its 5' and 3' ends respectively is eluted at 36.1 min.

$\epsilon$-Maleimidocaproic acid *N*-hydroxysuccinimide ester (MHS) (4.5 $\mu$moles) in 50$\mu$L DMF and peptide (5 $\mu$moles) were incubated in 1mL 0.1M phosphate buffer , pH 6.5[14] + 0.3M NaCl for 1 hour at room temperature. Under these conditions we obtained 46% of $\alpha$-monosubstituted peptide[15] (Table 1). The $\alpha$-monosubstituted peptide, isolated by HPLC had a retention time ($t_R$=25.5 min) (Table 2) and a UV spectrum revealing the presence of both Tyr and maleimide groups. Oligonucleotide 0.1 $\mu$mole bearing a thiol activated group (SS-Py) at its 3' end and a fluoresceinyl moiety at its 5' end was reduced with TCEP (0.15 $\mu$mole) in 500 $\mu$L 2M NaCl, 0.1M phosphate buffer, pH 7.2, for 1 h at room temperature under nitrogen.

**Table 1**: Substitution of the amino-groups of the Tyr-Lys-Asp-Glu-Leu peptide by MHS at different pH.

| pH | Time (min) | $\alpha$-substituted % | $\alpha,\epsilon$ disubstituted % | $\epsilon$-substituted % | Free peptide % |
|---|---|---|---|---|---|
| 6.5 | 10 | 19 | 0 | 0 | 81 |
| 6.5 | 60 | 46 | 8 | 2 | 44 |
| 6.5 | 180 | 55 | 15 | 5 | 25 |
| 7.5 | 10 | 38 | 12 | 2 | 48 |
| 7.5 | 60 | 47 | 17 | 4 | 32 |
| 7.5 | 180 | 48 | 21 | 6 | 25 |

The different peptides were analyzed and purified by reverse-phase chromatography on a LiChrospher 100RP-18, 5$\mu$m column (250x10mm). Mobile phase A was water + 0.1% ammonium acetate and mobile phase B was acetonitrile + 0.1% ammonium acetate. The gradient was 5% to 40%B in 25 min. The flow rate was 3 mL/min.

The coupling reaction between oligonucleotide-SH and $N_\alpha$-maleimidocaproyl-peptide was carried out at room temperature in 0.1M phosphate pH 7.2 + 2M NaCl for 5h. One equivalent of the oligonucleotide was added to 1 equivalent of peptide without elimination of the TCEP excess. The oligonucleotide-peptide conjugate ($t_R$=22.0 min) (Table 2) was collected (yield 82%). After purification and desalting on Biogel $P_2$ the conjugate had the expected spectral characteristics related to both DNA and fluorescein moieties and the expected amino acid composition: Tyr(0.81), Lys(1.22), Asp(0.93), Glu(1.10), Leu(1.00); small amounts of glycine and other amino compounds deriving from oligonucleotide degradation during acidic hydrolysis were also detected. Electrospray mass spectrometry confirmed the molecular weight calculated for the oligonucleotide-peptide conjugate.

The strategy developped in this paper allows to link covalently an oligonucleotide to an unprotected peptide or a protein through a stable, no reducible, thioether bond. The coupling reaction occurs in aqueous solution at pH 6.5 under mild conditions where the maleimide is quite stable and the $\epsilon$-amino group is poorly reactive.

**Table 2** : Retention times of the synthetized compounds.

| Products | Retention time (min) |
|---|---|
| Tyr-Lys-Asp-Glu-Leu | 8.4 |
| $N_\alpha$-maleimidocaproyl-Tyr-Lys-Asp-Glu-Leu | 25.5 |
| Ftc-oligonucleotide-SH | 18.0 |
| Ftc-oligonucleotide-Tyr-Lys-Asp-Glu-Leu | 22.0 |

The different products were analyzed and purified by reverse-phase chromatography on a LiChrospher 100RP-18, 5$\mu$m column (250x10mm). Mobile phase A was 0.1M triethylamine acetate buffer (TEAA) (pH 7.0) + 5% acetonitrile and mobile phase B was acetonitrile + 5% 0.1M TEAA. The gradient was from 5% to 30%B for 30 minutes. The flow rate was 3mL/min.

This strategy does not require a selective protection of the $\epsilon$-amino group of lysyl residues during the coupling and consequently a deprotection step of the conjugate. This last step would hydrolyze the oligonucleotide moiety if it is done in acidic medium. Finally the use of tris (carboxyethyl) phosphine as a reducing agent, allows the preparation of the oligonucleotide peptide conjugate without withdrawal of the excess of TCEP and without any purification step of the intermediate oligonucleotide mercaptan avoiding oligonucleotide dimerization. The intracellular traffic and biological efficiency of the covalent oligonucleotide-peptide conjugates synthetized are currently under investigation. Several oligonucleotide-KDEL conjugates specific for human immunodeficiency virus (HIV) genomic sequences have been prepared by using the above described method.

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15. In order to identify the three MHS-substituted pentapeptides, the reaction product was incubated with aminopeptidase M in a final volume of 400 $\mu$l of tris/HCl buffer (pH 7.2) for 1h at 37°C. The reaction mixture was analyzed by HPLC as described in Table 1. The native peptide and the $\epsilon$-substituted peptide were completly hydrolyzed by the aminopeptidase, whereas the $\alpha$ substituted and $\alpha,\epsilon$ disubstituted peptides were not.

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