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Selective Coupling of a Highly Basic Peptide to an Oligonucleotide

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Abstract: A highly basic peptide (net charge +8) derived from the HIV-1 Tat protein is conjugated with quantitative yield within minutes to a 19 mer rhodamine-labelled phosphodiester oligonucleotide activated by the pyridine sulfenyl group. To avoid precipitation due to antagonist charges of the oligonucleotide and the peptide the conjugation was performed with high salt concentration (400mM) and acetonitrile (40%). © 1997, Published by Elsevier Science Ltd. All rights reserved.

One possibility of regulating gene expression *in vivo* is the use of synthetic oligonucleotides (ODN) binding specifically to complementary DNA or RNA targets. Efficient cell internalization and adequate intracellular targeting of oligonucleotides remain major problems in most cases. Several strategies are currently explored in order to increase cytoplasmic or nuclear delivery. One of these consists in covalent binding of ODN to peptides able to translocate through the plasma membrane or to destabilize the endosomal membrane such as the Antennapedia third helix homeodomain peptide[1] or the N-terminal peptide of Influenza virus Hemagglutinin[2] respectively. In similar fashion the conjugation of an oligonucleotide to poly-(L-Lysine) increased its biological activity by two orders of magnitude as compared to the unmodified oligonucleotide.[3] Ongoing work[4] has focused on the use of a highly basic peptide domain (a cluster of eight basic amino-acids located within a short sequence of nine residues) from HIV-1 Tat, a protein known to translocate efficiently into cell nuclei.[5,6] It is anticipated that such a basic peptide would favor binding to the plasma membrane phospholipid bilayer and allow cell uptake. A labile disulfide bond has been introduced between the peptide and the ODN in order to allow the intracellular delivery of the free oligonucleotide in a prodrug type of strategy.

Several protocols for the specific coupling of a peptide to an ODN have been previously described. These include the reaction of a SMCC-activated ODN,[2] a maleimide-activated ODN[7] or an iodoacetylated ODN[8] reacted with an excess of a peptide harboring a cysteine residue. Alternatively a maleimide derivatized peptide[9] or a Nα-(bromoacetyl) modified peptide[10] was reacted with a thiol function of the oligonucleotide. However, all these strategies lead to the formation of a stable bond between the peptide and the ODN. This type of stable link is not required when the peptide is expected to act essentially as a carrier for the intracellular delivery of an oligonucleotide. To induce the formation of a specific disulfide bond, a strategy using a pyridine sulfenyl group has been used for the heteroconjugation of two peptides.[11,12] A similar approach was described for the coupling of an ODN to a peptide.[13] In this latter case, the activated thiol function was carried by a short peptide corresponding to the nuclear transport sequence from the SV 40 large-T antigen and the coupling reaction was performed overnight with a tenfold excess of ODN.

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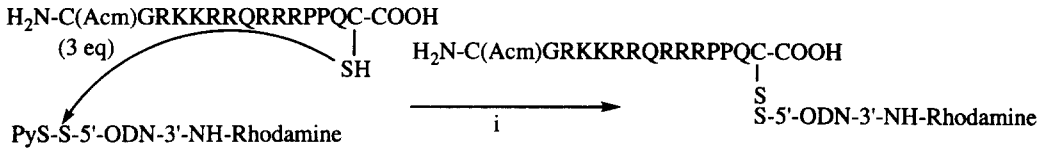
In the present paper, the sulfhydryl activated function is carried by the ODN.[14] Regarding the current tendency to develop new ODN analogues, this strategy is interesting in allowing the coupling of the totality of the ODN activated with the sulfenyl pyridine function in the presence of a low excess of the peptide. Indeed a quantitative coupling of the peptide to the ODN was obtained within a few minutes when the reaction was performed with a threefold excess of the free sulfhydryl peptide[15] (Fig.1).



i) 40% CH_3CN, 400 mM KCl, room temperature

Fig.1: Reaction scheme for the ODN-peptide conjugation

The pyridine sulfenyl-activated thiol function on the 5' end of the ODN allowed the quantitative formation of a single heterodimeric form of the peptide-ODN conjugate by nucleophilic attack of the free sulfhydryl group at the C-terminal end of the peptide. Because of the high level of positive charges of the peptide and the negative charges of the ODN, precipitation occurs during the coupling reaction. This problem was overcome by addition of salts and acetonitrile to neutralize charges and to help solubilization. An aliquot corresponding to 10 OD units (60 nmoles) of a purified rhodamine-labelled ODN was adjusted with KCl and acetonitrile to final concentrations of 400 mM and 40% (V/V) respectively. Before addition of the peptide the availability of the cysteine residue sulfhydryl group was monitored by its absorbance at 412nm after reaction with 2-4,dithio(bis)nitrobenzoic acid[16]. It is worth noting that no oxydation of this peptide was ever observed probably because of charge repulsion due to the high number of basic residues. Nethertheless nitrogen was bubbled in the ODN solution before addition of the peptide. A threefold excess of the peptide (180 nmoles) was then slowly added to the ODN solution. The reaction was monitored by HPLC using a DEAE column (Protein-Pak™, DEAE-15HR, 5x100, Waters) with a linear gradient from 20% to 60% in 20 min of 0.65 M KCl in 40% (v/v) acetonitrile and 10 mM NH_4Ac pH 7.2 and recorded at 220 nm and 254 nm with a λ-scan photodiode array from 200 nm to 560 nm. The newly formed conjugate elutes several minutes earlier than the free oligonucleotide under these HPLC conditions (Fig. 2a). This procedure also allows the elimination of the excess free peptide which eluted with the solvent front. Precipitation problems during reverse phase HPLC (RP-HPLC) resulting from the excess of peptide over the conjugated compound have been previously reported[8]. Desalting was achieved by precipitation of the conjugate with cold ethanol, followed by centrifugation and additionnal washing steps with 70% (v/v) ethanol in water. After drying at room temperature, the conjugate was resuspended in 10 mM NH_4Ac and its purity was assessed by RP-HPLC (gradient 10% to 50% of CH_3CN in 20 min) with dual absorbance recording at 254 nm and 215 nm using a Beckman System Gold 168 photodiode array detector (Fig. 2b). The λ-scan showed a λ-max at 254 nm and an absorbance peak at 550 nm corresponding to the rhodamine moiety. The composition of the peptide moiety of the conjugate was assessed by amino-acid analysis after 24h hydrolysis in 5.6N HCl (data not shown). Treatment of the conjugate with 10 mM DTT for 1 hour at 37°C led to the quantitative regeneration of the free oligonucleotide as assessed by ion exchange chromatography as described above (data not shown).

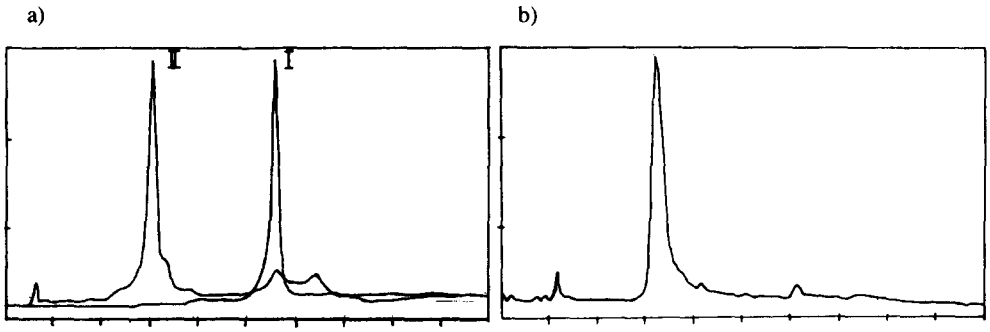


Figure 2: a) HPLC profile of the ODN (peak I) and of the crude conjugate (peak II). Chromatography was performed on a DEAE column using a linear gradient from 20 to 60% of 0.65M of KCl in 40% (v/v) acetonitrile in 20 min. b) RP-HPLC of the isolated conjugate. Detection for a) and b) was at 254 nm.

In conclusion, this coupling procedure appears particularly adapted for the quantitative coupling of highly basic peptides to nucleic acids, mainly when the amount of the ODN to be coupled is limiting.

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14. Tat-N ODN (sequence 5'-ggC-TCC-ATT-TCT-TgC-TCT-C-3') was provided by Eurogentec (Seraing, Belgium) with the following functionalization. ODN 3' end was modified using the C7 amino-modifier CPG from Glen Research (Sterling, VA) and 5' end was modified with the Thio-modifier C6 S-S phosphoramidite (Glen Research, Sterling, VA). The sulfhydryl group of the ODN was then activated by the supplier with 2,2'-dithiopyridine as previously described[10]. Before condensation to the peptide, the 3' end of the oligonucleotide was labelled with 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (5(6)-TAMRA, SE (Interchim SA, Montluçon, France) in 500 mM $NaHCO_3/Na_2CO_3$, pH 9. 50 OD units of ODN (320 nmoles) were reacted with a large excess of TAMRA,SE (5400 nmoles, 17 eq) dissolved in DMSO for 4h in the dark at room temperature. The labelled ODN was purified by HPLC using a linear gradient (10 to 30% for 30 min of CH_3CN in 10 mM NH_4Ac pH7.2) with dual absorbance detection at 254 nm and 550 nm. The yield of the labelling reaction was estimated to be 75% according to

HPLC peak integration. The purified labelled ODN fractions were pooled and concentrated by partial evaporation on a Speed-Vacuum microcentrifuge before the coupling reaction with the peptide.

15. Tat derived peptide was chemically synthesized by solid phase method as previously described (Vivès, E. & coll. *J. Virol.* **1994**, 68, 3343-3353). Only minor modifications were applied for this synthesis. i) The C-terminal cysteine side chain was protected by a *p*-methylbenzyl group and the N-terminal cysteine side chain by the HF stable protecting group acetamidomethyl (Acm). ii) The coupling reagent was BOP/HOBt. iii) The deprotection of the Nα-Boc group was performed in two steps in 100% trifluoroacetic acid with reaction times of 1 and 3 min successively. Peptide cleavage and HF-labile lateral-chain deprotection were achieved as described[8]. The peptide was purified by semi-preparative C18 reversed phase HPLC. Analytical HPLC was carried out on Hypersyl C18-5mm column (4.6x250). Peptide molecular weight was determined by Electrospray Ionization Mass Spectrometry (calculated: 1996.3 Da; found: 1996.5). Composition and quantification of the peptide were determined after hydrolysis of an aliquot for 24h at 110°C. Peptide was resuspended in PBS (pH 7.3) at a concentration of 10mg/ml and kept frozen until further use.

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