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Toward high yield synthesis of peptide–oligonucleotide chimera through a disulfide bridge: A simplified method for oligonucleotide activation

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Abstract—During the last decade, cell penetrating peptides (CPP) have been extensively used to mediate the cellular delivery of non-permeant biomolecules, including oligonucleotides (ONs). A covalent linkage between the CPP and the transported ON is required to mediate efficient cell internalization, and a disulfide bridge between the CPP and the ON has been shown to induce the most potent biological response. In this paper, we describe the activation. In a one step process of the sulfhydryl function from a synthon commercially available for ON synthesis. In addition, since the highly cationic nature of currently used CPP caused serious precipitation problems during the coupling step, we further improved the method by adsorbing the crude activated ON on an anion exchange matrix prior to specific peptide coupling.

Synthetic oligonucleotides (ONs) have interesting therapeutic potentials in various biological applications.[1] More recently, the expression of a targeted protein was reduced impressively by small interfering RNAs (siRNA).[2] Since ONs are poorly taken up by cells, several transfection methods have been developed. However, most of these techniques are not exempt of undesired effects such as toxicity and immunogenicity.

About 10 years ago, several small peptides which are able to pass through the plasma membrane were discovered.[3,4] Interestingly, these peptides, named cell penetrating peptides (CPP) or protein transduction domains (PTD),[5,6] improve the cellular delivery of cargoes such as peptides and proteins,[5,6] drugs,[7] microorganisms[8,9] or nucleic acids.[10,11] For most of the CPP, a covalent bond with the cargo is required to promote transfection, and the coupling of CPP to nucleic acids is very challenging because of incompatibilities in their respective chemistry.[12–15]

Disulfide bond formation between the ON and the CPP has been extensively used[14–19] since the disulfide bridge is supposed to be stable in the extracellular medium while labile in the reducing intracellular environment. Thus, after behaving as a 'Trojan' peptide[20] for allowing the cellular ON entry, the ON is expected to be released from the CPP by the cytosolic glutathione. Reduction of the disulfide linkage has been documented for a CPP–ON chimera in cell extracts[21] and also in intact cells following CPP-mediated delivery of a cargo peptide.[22] In keeping with efficient release, a morpholino-ON derivative was shown to fulfill its expected biological effect with a higher efficacy when conjugated to a CPP by a disulfide linkage than when coupled through a stable bond.[11] The formation of disulfide bond between the peptide and the ON exists in two distinct flavors: the use of bifunctional cross-linkers[11] and the pre-activation of a sulfhydryl group carried by one of the chimera components.[14,17–19,23–25] Incorporation of a sulfhydryl function at either the 3′ or 5′ end of the ON is usually performed by two main routes. First, a disulfide bridge can be incorporated during the ON synthesis using various thiol-modifiers. Then, a reduction step has to be carried out with reducing agents such as dithiothreitol (DTT).[15,24] Alternatively, a sulfhydryl group can be

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directly incorporated by substituting a carbonyldiimidazole with cystamine.[25] In both cases, however, the subsequent activation of the thiol function with, for instance, a nitropyridine sulfenyl moiety, is required.[14,15,24,25]

A thiotrityl synthon (Trt-S) is commercially available for the 5′-ON modification. To date, to release the trityl group and to restore the free thiol function, a silver nitrate treatment is required. Then, the excess of silver nitrate is precipitated with DTT and free ON-thiol is recovered by gel filtration. The thiol group is subsequently activated as described above. Whatever be the routes, published strategies require several time-consuming steps often leading to loss of material and more often to poor yields.[14]

The direct substitution of the trityl group by a thio nitropyridine has been used in peptide chemistry[26] but, to our knowledge, not evaluated in ON synthesis. In this report, we describe the quantitative one-step substitution of the thiotrityl-protecting group by a thionitropyridine group. We investigated this substitution on unmodified ON as well as on phosphorothioate and 2′*O*-methyl ON analogues (Scheme 1).

Interestingly, the activated ON can be directly adsorbed onto an anion exchange matrix from the crude activation medium as previously described,[24] thus avoiding an additional purification step and allowing the 'in batch' use for large scale-up process. A Tat peptide–ON chimera was directly and quantitatively obtained following this protocol.

The oligonucleotide Trt-S–$(CH_2)_6$–d(GAAGGGATTT CCC-TCC) (1 μmol scale) was assembled on a Applied Biosystems 381A DNA synthesizer using the phosphoramidite chemistry.[27] At the end of the ON assembling, 6-(*S*-trityl-6-mercaptohexyl)-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite (Trt-S–$(CH_2)_6$–5′-Thiol Modifier C6, Eurogentec, Belgium) (18 μmol) was incorporated. The usual oxidation step was performed in 0.02 M iodine in THF/pyridine/water to avoid additional Trt-S oxidation. Cleavage was then performed with three washes (1 mL) of a 28% ammonium hydroxide solution during 90 min and deprotection was allowed for 4 h at 55 °C. The crude ON was further purified by reversed-phase HPLC using a 20 min linear gradient of 8–40% in 50 mM TEAAc (pH 7). Homogeneous fractions were pooled and freeze-dried successively 10 times to fully remove TEAAc salts. Trt-S–ON was then kept in water (0.1 OD_{260nm} per μl) at −20 °C until further use.

Direct substitution of the trityl protection by the nitropyridine sulfenyl group was then performed with the corresponding chloride in solution on unmodified Trt-S–ON phosphodiester[28] (Fig. 1A) or Trt-S–phosphorothioate and Trt-S-2′-*O*-methyl ON analogues (data not shown). In all cases, the reaction was completed within 1 h with total substitution of the starting ON (Fig. 1B). The HPLC peak corresponding to the substituted phosphodiester ON (13.76 min) was collected (Fig. 1C) and analyzed by MALDI-TOF spectrometry.[29] Two signals were however observed: one corresponded to the expected molecular weight of the NpySS–ON (calculated: 5416.69; measured: 5416.81) (Fig. 2) along with another signal with an apparent mass of 5263.27. The latter one corresponds to the reduced,

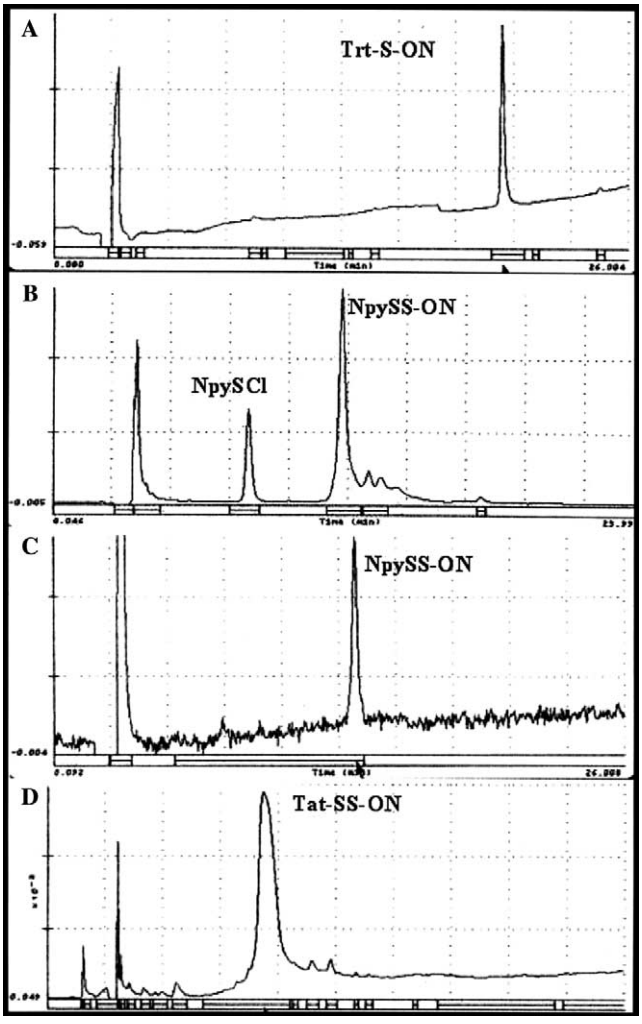
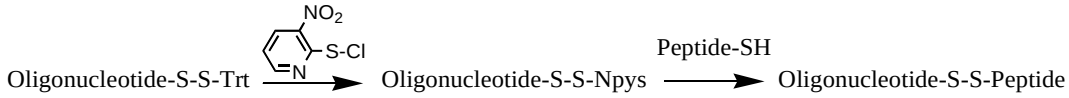


Figure 1. HPLC elution profiles of the different ONs and conjugates. (A) Trt-S–ON after purification (t_R = 20.24 min); (B) crude activation mixture of the Trt–ON; (C) purified NpyS-activated ON (t_R = 13.76 min); (D) Tat–SS–ON conjugate after elution from the DEAE-beads (t_R = 9.96 min). All purified compounds were further characterized by MALDI-TOF analysis (see Fig. 2). Signals were recorded at 215 nm excepting for the HPLC profile C (260 nm).



Scheme 1. Preparation of the ON–CPP conjugate.

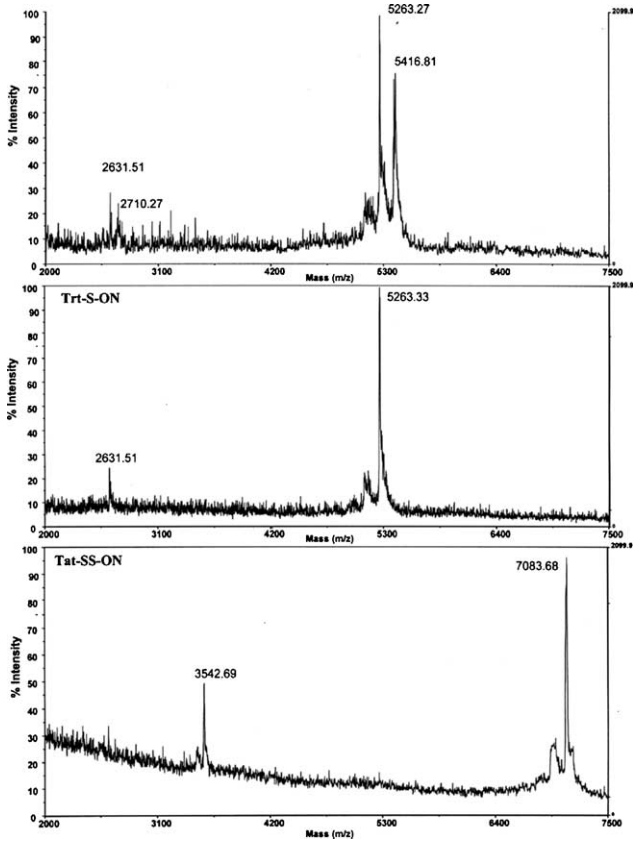


Figure 2. MALDI-TOF analysis of the different ONs. Top: purified NpyS-activated ON showing two signals: $[M-H]^{-1}$ = 5263.27 and: $[M-H]^{-1}$ = 5416.81. Middle: NpyS-activated ON after DTT reduction $[M-H]^{-1}$ = 5263.33. Bottom: Tat–SS–ON conjugate after elution from the DEAE-beads $[M-H]^{-1}$ = 7083.68.

and therefore unactivated, ON–SH, meaning that the NpyS group was partially released. Whether the cleavage occurred during laser exposure or was due to an undesired reaction leading to the reduced ON eluting with an identical retention time during the HPLC purification was assessed as follows. The crude activated NpySS–ON was submitted to a reduction in a 10 mM DTT solution to obtain a sample of ON–SH. The reaction was monitored by HPLC. The peak corresponding to the activated ON disappeared, and a new peak with a much lower t_R (10.28 min) was collected and analyzed by MALDI-TOF spectrometry (Fig. 2, middle). The MALDI-TOF spectrum showed a signal with an experimental mass $[M-H]^{-1}$ of 5263.40, which demonstrates that the ON–SH and the NpySS–ON have different retention times and cannot be collected together from HPLC purification. Therefore, the apparent MALDI-TOF analysis contamination resulted from the laser-mediated cleavage of the disulfide bridge. This may result from the peculiar reactivity of pyridine and phenyl disulfide compounds toward laser photo irradiation.[30,31] Thus, the Trt substitution yielded quantitatively to the NpyS-activated ON since all the Trt–ON was substituted (Fig. 1B).

We initially performed the purification of the activated ON by HPLC. However, it was observed that some material was lost during the purification process. We avoided this purification step by adsorbing directly the activated NpySS–ON from the crude mixture on an anion exchange matrix after pH neutralization with triethylamine following resuspension of the reaction medium in 50 mM TEAAc, pH 6.8. The mixture was then transferred on 300 μl DEAE beads previously washed and equilibrated with 50 mM TEAAc solution (pH 6.8). The choice of these pH conditions was directed by the further heterodimerization step with the peptide-SH. Surprisingly, ON adsorption was not completed after some hours of agitation as assessed by easily monitoring the optical density of the supernatant at 260 nm. Some ONs were still unbound after 7 h. Therefore, the ON was routinely allowed to bind to the matrix under weak agitation overnight to yield to its quantitative adsorption. Once the activated-ON was fully adsorbed, five resin washes with 500 μl TEAAc solution were performed to remove excess of activating reagent. HPLC monitoring of the washing solutions confirmed the complete removal of the excess of Npy-SCl and the released thiotrityl by-product (data not shown).

Eventually, Tat peptide (2 equiv, assembled and purified as previously described[4]) was added to the resin beads resuspended in a minimal volume of TEAAc 50 mM pH 6.8. The advancement of the reaction was monitored by HPLC. Some unconjugated ONs were still found after 4 h of incubation; therefore, an overnight incubation was performed to yield to complete conjugation (Fig. 1D). Such an overnight coupling has also been recently performed when condensing Tat peptide to a methylphosphonate ON[25] or an Arg-rich peptide to an ON.[24] The resin was washed twice with 400 μL TEAAc 50 mM, pH 6.8, to remove the uncoupled Tat peptide and then three times with 200 μL of a 2 M KCl solution to desorb the conjugate. The supernatants were pooled and the resulting solution was desalted after loading on a C18 Sepak cartridge (Waters Millipore), washed with water to remove salts, and eluted with a final water/acetonitrile solution (50/50 v/v). The chimera was then analyzed by HPLC, MALDI-TOF, and quantified by optical density measurement. Monitoring the reaction advancement was performed by sampling small amounts of the resin and by washing several times with 2 M KCl to elute the adsorbed chimera from the cationic matrix. The Tat–SS–ON conjugate was quantitatively obtained and further fully characterized by HPLC analysis (Fig. 1D) and by MALDI-TOF analysis (Fig. 2, bottom; $[M-H]^{-1}$ 7083.68). It is noteworthy that no uncoupled Tat peptide was found in the solution eluted from the resin during the desorption phase. This has been fully assessed by the absence of HPLC or MALDI-TOF signal corresponding to the Tat peptide itself (data not shown).

The covalent coupling of an ON to a peptide through a disulfide bridge is increasingly considered to be essential for the peptide-mediated delivery of ON, as shown recently for the delivery of morpholino-ON analogues.[11] For biological studies, large amounts of peptide–ON chimera will probably be required and this one-step activation method presents two main

advantages. First, it uses a thiotritylated moiety, which is commercially available and easily introduced during ON synthesis. Moreover, the incorporation of a thiotrityl group associated with a capping step during the ON synthesis is known to help in the ON purification by reversed-phase HPLC because of its high hydrophobicity. Second, the activation method performed on the purified Trt-S–ON allows the quantitative 'batch' conjugation of peptides even with a high cationic content through an intracellular reducible bond.

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28. Trt-S–ON (typically 7 μL of a 0.1 OD per μL, 0.7 OD; 4.16 nmol) was dried in a SpeedVac unit and resuspended in 10 μL of glacial acetic acid. 8.3 μL of a 10 mM solution of 3-nitro-2-pyridinesulfenyl chloride (Cl-NPyS) (8.3 nmol, 20 molar excess) was then added and the reaction was allowed for 1 h. The reaction was monitored by HPLC with a 20 min linear gradient of 12–20% acetonitrile in 50 mM TEAAc, pH 7.2. Prior to HPLC purification, the crude reaction media were resuspended in 500 μL of 50 mM TEAAc and loaded with a manual injector equipped with a 1 mL loop. Analytical HPLC characterization of the activated-ON was performed in the same eluting conditions.
29. MALDI-TOF mass spectra were recorded on a negative mode on a Voyager (Perseptive Biosystems, Framingham, MA, USA) mass spectrometer equipped with a N_2 laser (337 nm). MALDI conditions were: accelerating potential, 24,000 V; guide wire, 0.05% of accelerating voltage; grid voltage, 94% of accelerating voltage; delay extraction time, 550 ns. The ONs (0.01–0.05 OD_{260nm}) were solubilized in 8–10 μL of the matrix 2,4,6-trihydroxyacetophenone (THAP, 45 mg, ammonium citrate, 4 mg) in 500 μL acetonitrile–water (50/50 v/v). The resulting mixture (1 μL) was spotted on the stainless steel probe tip and left to dry in air. The samples were then subjected to MALDI-TOF analysis.
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