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A mild method for fluorescein labeling of base-sensitive oligonucleotides on solid support

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Abstract

A simple and mild method is described to covalently label base-sensitive supported oligonucleotides, such as prooligonucleotides, with unprotected carboxyfluorescein as a highly reactive dimethylamino-pyridinium ester generated in situ. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: fluorescein; base-sensitive oligonucleotides.

Replacement of radioactive labeling of biomolecules such as peptides and oligonucleotides by fluorescent dyes is gaining increasing interest for the investigation of biological materials.[1,2] One of the most common tags, carboxyfluorescein (CF), is now widely used in DNA chemistry and biology, making possible a large panel of structural studies and cell imaging, such as DNA hybridization monitoring by Fluorescence Resonance Energy Transfer[3] or variation of fluorescence anisotropy,[4] DNA sequencing,[5,6] intracellular localization, and even quantification of fluorescent oligonucleotides by means of fluorescence microscopy and flow cytometry.[7]

Numerous strategies have been designed for the labeling of oligonucleotides with protected or unprotected fluorescein: pre- and post-synthetic routes, as well as automated solid-phase synthesis.[2] In most cases, these methods are not compatible with base-sensitive oligonucleotides such as SATE prooligonucleotides (prooligos),[8] which are constituted with thioester functions and phosphotriester internucleosidic linkages (Fig. 1).

Common phosphoramidite dyes of CF (usually protected with pivaloyl groups),[9,10] as well as the well-known fluorescein isothiocyanate (FITC) used for coupling in aqueous solution at pH 9–10 and many other techniques, are not available in our case, since they usually need basic conditions, which are incompatible with the integrity of SATE prooligonucleotides. Likewise, Weber et al.[11] described the labeling of supported peptides following different procedures to activate the 5,6-carboxylic function of CF: pre-activation of CF with hydroxysuccinimide

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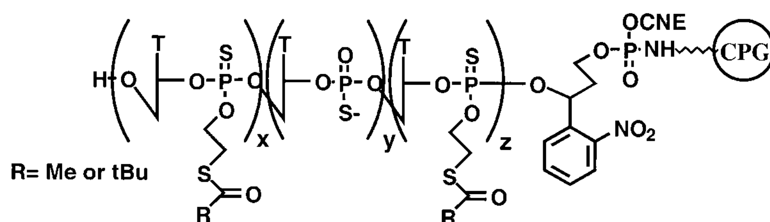

Figure 1. Prooligonucleotide bound to a photocleavable solid support

(HOSu) did not lead to a sufficiently reactive ester, but in situ activation with DIC/HOBt gave high coupling yields (>90%). In the case of prooligos, the presence of phosphorothioate diester leads to side reactions and makes the in situ activation with any carbodiimide impossible.

We present in this paper a new, simple and mild method to covalently label base-sensitive supported oligonucleotides such as prooligos with unprotected CF.

Our first attempt to label the prooligos with the succinimide active ester of CF (CF-OSu, **1**) gave very low coupling yields (15–25%). For that reason, we decided to suractivate CF-OSu in situ by adding DMAP ($pK_a = 9.7$) in anhydrous DMF (Fig. 2).

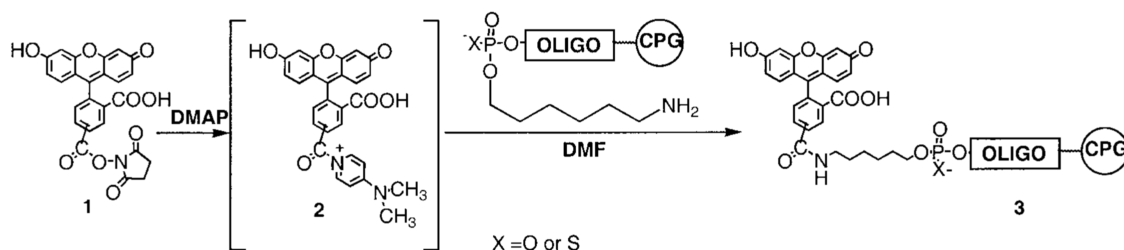

Figure 2. Post-synthetic coupling on the C6-amino linked prooligonucleotide

CF-OSu (**1**) was obtained following a classical procedure: CF (1.0 eq.), DCC (0.95 eq. to ensure totally conversion to DCU), and HOSu (1.0 eq.) were stirred overnight at room temperature in a sufficient volume of dry DMF to obtain a 0.1 M concentration. After elimination of DCU by filtration, the solution was ready for use and did not need further purification.[12] The prooligos, assembled on a 381A ABI synthesizer[13] on a photolabile solid support,[14] were functionalized quantitatively with the amino-linker *N*-monomethoxytritylaminohexyl-*H*-phosphonate.[15,16] After oxidation of the *H*-phosphonate diester (I_2/Py/H_2O or S_8/Et_3N/DMF), the MMtr group was removed by a 6 min TCA (3% in CH_2Cl_2) treatment followed by CH_3CN/Py washing. To monitor the efficacy of each step, small amounts of prooligo were cleaved from the solid support after oligo synthesis, functionalization and detritylation, and analyzed by reverse phase HPLC and MALDI-TOF MS.

An optimized labeling procedure was set up on various 1 μmol scale supported prooligos bearing either *t*Bu- or MeSATE groups. Labeling was performed manually during 3 h by percolation with two syringes using 400 μl of the 0.1 M solution of CF-OSu in dry DMF and 0.5 eq. DMAP with respect to CF-OSu. In all cases, coupling yields were between 70 and 85% (HPLC monitoring; Fig. 3). The use of 1–2 eq. DMAP or a more concentrated solution of CF-OSu (0.3 M), as well as extended coupling time, did not drive the labeling to completion, but led in the case of MeSATE prooligos to the loss of some MeSATE groups (up to 20%). Catalytic amounts of

DMAP (below 0.1 eq.) decreased the global reaction efficiency, probably because of the weak stirring conditions and kinetics parameters (under 0.1 eq. of DMAP, compound **2** is no longer stoichiometric with the 1 μmol supported prooligo). CPG beads were thoroughly washed with DMF, H_2O and CH_3CN. Finally, prooligos were cleaved by photolysis in CH_3CN/H_2O,[17] analyzed and purified by C_{18} reverse phase HPLC to yield pure labeled prooligos characterized by MALDI-TOF MS. Fig. 3 shows HPLC profiles of crude (A) and purified (B) MeSATE dT_{20} prooligo ($x=0$, $y=5$, $z=15$) at 260 and 494 nm. The peak at 33 min (Fig. 3A) corresponds to the unlabeled 5′-amino-linked prooligo, as confirmed by MALDI-TOF analysis.[18]

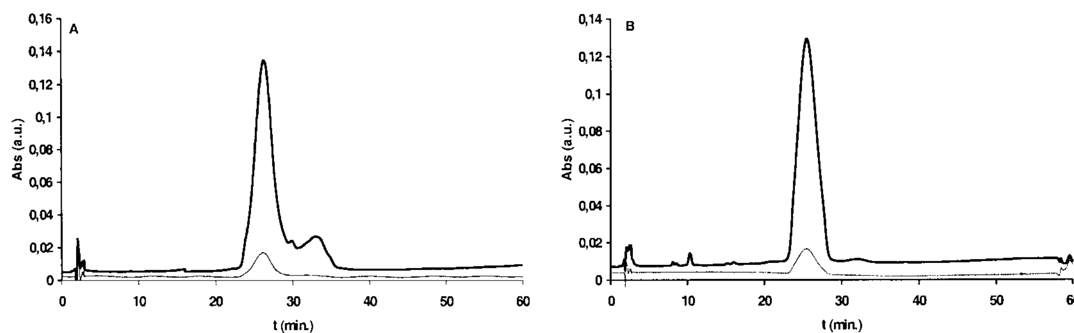

Figure 3. HPLC profile of crude (A) and purified (B) fluorescein labeled MeSATE dT_{20} ($x=0$, $y=5$, $z=15$) at 260 and 494 nm

Our attempts to generalize this method to classic oligonucleotides gave only moderate labeling yields (60%).

1. Fluorescence properties of carboxyfluorescein-conjugated prooligonucleotides

It is known that conjugation to nucleic acids alters the properties of fluorescein, especially if one considers the modifications of the local electrostatic potential[19] and the general environment of the fluorescence moiety by masking the negative charges with more lipophilic substituents.[1] To interpret further biological experiments, such as cell uptake study and fluorescence quantification using flow cytometry, we compared the fluorescence properties of an MeSATE dT_{20} prooligo ($x=6$, $y=7$, $z=7$; Fig. 1) with its dT_{20} phosphorothioate (dT_{20} PS) analogue as a function of pH.

Fluorescence excitation profiles of the dT_{20} PS and of the MeSATE dT_{20} prooligo showed similar spectral shifts (data not shown), but different excitation maxima intensities, which reflects the intensity of fluorescence emission.

Thus, emission spectra (Fig. 4) measured at various pH values between 1.5 and 7 showed, as expected, decreasing fluorescence intensities for decreasing pH values, since CF exists in several protolytic forms: from the intensive fluorescent dianion and fluorescent monoanion present around pH 7, to other non-fluorescent protonated forms at more acidic pH. However, a dramatic difference was observed concerning the relative quantum yield of CF conjugated to the MeSATE prooligo in regard to the dT_{20} phosphorothioate: at pH 7, the prooligo exhibited half the fluorescence of its corresponding dT_{20} PS. Moreover, subsequent dependence on an acidic pH was found to be higher for the prooligo since, at pH 5, the fluorescence intensity of the SATE prooligo was 15-fold lower than that of the dT_{20} PS.

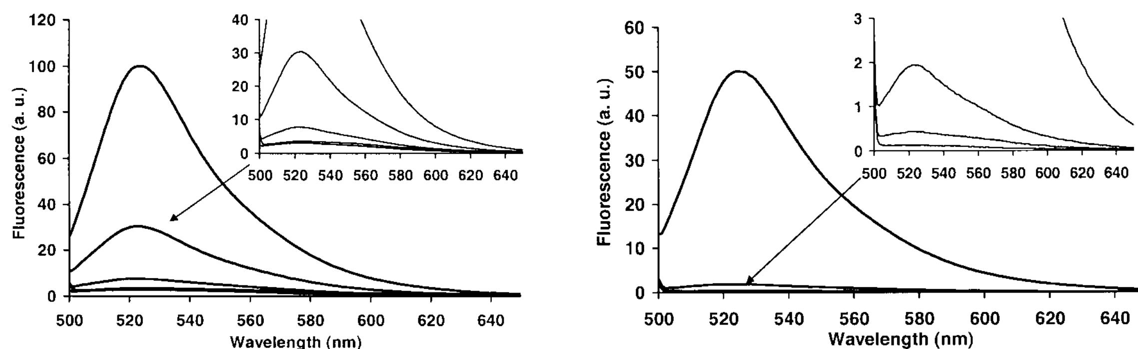

Figure 4. Emission spectra of fluorescein labeled dT_{20} PS (left) and MeSATE prooligo (right) (0.5 μM): top to bottom, pH 7, 5, 4, 3 and 1.5 (excitation wavelength = 494 nm)

To confirm the crucial influence of such modification, MeSATE groups were totally removed by aqueous ammonia treatment. The results of fluorescence intensity measured thereafter were exactly the same as those obtained for the dT_{20} PS (data not shown), proving the role of SATE groups in the fluorescence quenching.

Such decreases of the fluorescence intensity are often observed when the fluorescent dye is in a hydrophobic environment, for example through vectorization with vehicles such as lipofectin or other polymers,[7] and in our case by replacement of negative charges with neutral SATE groups. Furthermore, variation of the fluorescence intensity relation between both compounds as a function of pH might be explained by an increase in the pK_a of CF when conjugated to the prooligonucleotide. Indeed, a pure solvatation effect could not explain such a difference of the fluorescent behavior. It is more likely that CF when linked to the prooligonucleotide was already in the protolytic form (poorly fluorescent) at pH 5, while this protolytic form of CF when linked to the T_{20} PS occurred around pH 4.

In conclusion, we have described a mild method for labeling base-sensitive oligonucleotides with unprotected CF on a solid support, using a highly reactive dimethylaminopyridinium ester generated in situ. This method might be further applied to label any sensitive supported oligonucleotide under mild conditions.

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