ORGANIC LETTERS

2011
Vol. 13, No. 8
2000–2003

Synthesis of the 5′-Se-Thymidine Phosphoramidite and Convenient Labeling of DNA Oligonucleotide

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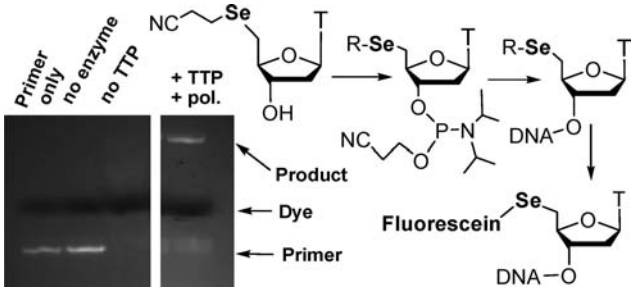
Received February 12, 2011

ABSTRACT



The synthesis of the 5′-Se-derivatized thymidine and phosphoramidite is reported, and the Se-phosphoramidite was incorporated into DNA. Because of its high nucleophilicity, this 5′-Se-moiety permits convenient postsynthetic modification of oligonucleotides. The proof of principle was successfully demonstrated by incorporating fluorescein into DNA. It was also found that the 5′-dye-labeled Se-DNA can be recognized by DNA polymerase as an efficient primer. This novel strategy opens a new avenue for nucleic acid probe preparation and detection.

Labeled nucleic acids (DNAs and RNAs) are useful for biochemistry, molecular biology, life sciences, and drug discovery.[1] The classical approaches for nucleic acid labeling include radioactive labeling, noncovalent intercalator binding, such as staining with fluorescent dyes (e.g., ethidium bromide or oxazoles),[2] and covalent nucleic acid labeling with a fluorescent dye at the sugar, phosphate, or nucleobase.[3] Since several dye-labeled phosphoramidites are commercially available,[4] DNA and RNA can be labeled during the solid-phase synthesis by using these expensive dye-modified phosphoramidites. Alternatively, the postsynthetic modification is another approach, where nucleic acids are first modified with a nucleophilic functionality (such as an amino or thiol group[5]) during the solid-phase synthesis, followed by a reaction of the nucleophile with an activated label (such as a dye) containing a good leaving group (e.g., bromo or iodo group). Since the incorporated label is not exposed to the chemical synthesis conditions (such as oxidant, acid, and base treatments), the postsynthetic modification can allow incorporation of much broader functionalities, especially labile ones.[6]

Although tremendous progress has been made in the postsynthetic modification of nucleic acids,[7] several challenges remained. For instance, its selectivity may be compromised due to the presence of many other nucleophiles (e.g., phosphate groups) in nucleic acids. Furthermore, its labeling efficiency is generally low due to the rapid deactivation of the activated labels (e.g., Br-activated dye[8]) in

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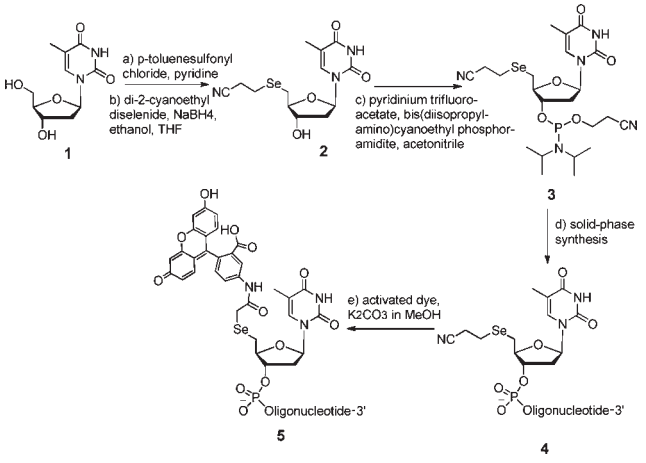
10.1021/ol200397c
Published on Web 03/24/2011

aqueous solutions. Thus, there are increasing demands in developing novel strategies for the postsynthetic modification. Since a selenol (R-SeH) is an excellent nucleophile (better than amino or thiol group), we expected that a selenol might facilitate the labeling and increase both the selectivity and efficiency. Therefore, we decided to incorporate a selenium atom onto the 5′-position of an oligonucleotide by replacing the 5′-oxygen atom (Scheme 1).

Our laboratory pioneered the atom-specific substitution of oxygen with selenium for the structural and functional studies of nucleic acids.[9] Our previous investigations of the selenium replacements at the base, sugar, or backbone indicate that the modified oligonucleotides did not show significant structural and functional perturbations after the selenium derivatization.[10] Since the 5′-terminal labeling is unlikely to cause any perturbations in a duplex and a polymerase extension reaction, we replaced the 5′-oxygen with selenium, offering a selenol as the nucleophile for the labeling. We describe here a straightforward synthesis of the 5′-Se-modified thymidine, its corresponding Se-phosphoamidite, and the Se-DNA. We have also demonstrated the proof of principle by showing that a fluorescent dye can be successfully incorporated into the DNA via the 5′-Se-functionality. Furthermore, we found that DNA polymerase can efficiently recognize the dye-labeled Se-DNA.

Scheme 1. Synthesis of the 5′-Se-DNA and Labeled-DNA



The synthesis of this novel 5′-Se-thymidine started from the activation of thymidine (**1**) at the 5′ position by a toluenesulfonyl group[11] (Scheme 1). This activating group was substituted by the cyanoethyl selenide, which was generated by reduction of di-2-cyanoethyl diselenide.[12] The protected Se-functionality was introduced in 89% yield, followed by converting **2** into its corresponding phosphoramidite (**3**, ^{m}T) in 80% yield. This 5′-Se-derivatized phosphoramidite (^{m}T) was then incorporated into oligonucleotide chemically on solid phase. The ultramild phosphoramidites were used for the synthesis because of the requirements of the Se-functionality deprotection and the labeling.[10,13] The coupling yield of this Se-modified phosphoramidite was approximately 95% on the basis of the shorter oligonucleotide study. After the full deprotection and dye labeling under a mild condition (MeOH solution of K_2CO_3),[10,13] the synthesized dye-labeled and nonlabeled DNAs were analyzed by HPLC, UV, and MS (Figures 1−3, S9−12 in Supporting Information) to confirm their integrity.

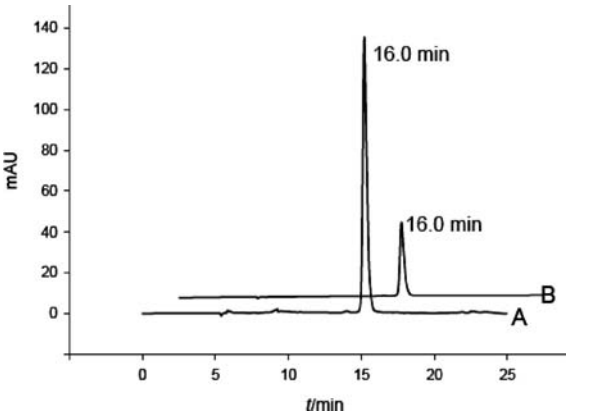


Figure 1. HPLC analysis of fluorescent dye-labeled DNA-22mer (5′-^{m}T-GCGTAATACGACTCACTATAG-3′). This analysis was performed on a Zorbax SB-C18 column (4.6 × 250 mm) with a linear gradient from Buffer A (20 mM triethylammonium acetate, pH 7.1) to 60% Buffer B (50% acetonitrile, 20 mM triethylammonium acetate, pH 7.1) in 20 min. Profile A was monitored under 260 nm, and Profile B was monitored under 490 nm. Retention time: 16.0 min.

To simplify the labeling strategy, it is desired to achieve the simultaneous labeling and deprotection in one pot, i.e., using the deprotecting reagent and the activated label (e.g., dye) at the same time. Before finally establishing the labeling strategy, we attempted several approaches and reagents, including stronger bases [e.g., NH_3 and 1,8-diazabicycloundec-7-ene (DBU)] and various polar solvents (e.g., DMF, DMSO, and MeOH). The activated dye was deactivated by a reactive deprotecting reagent (a stronger base), which might be the main reason that our early one-pot trials failed. On the other hand, the protecting groups of the nucleobases will not be completely removed, if a less-reactive deprotecting reagent (a weaker base) is used. Later, we found compatible conditions for

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both the deprotection and labeling in one step, when the ultramild phosphoramidites were used.[13] Because the 5′-Se-functionality is protected with a base-labile group, we can simultaneously remove the protecting groups of the Se-moiety and nucleobases. The generated selenol (R-SeH) or selenide anion ($R\text{-}Se^-$) is then alkylated by the activated dye in the reaction. Finally, we achieve the postsynthetic modification by just adding the activated dye (e.g., 5-Iodoacetamido-fluorescein, 5-IAF) during the deprotection step, easily resulting in the dye-labeled DNA.

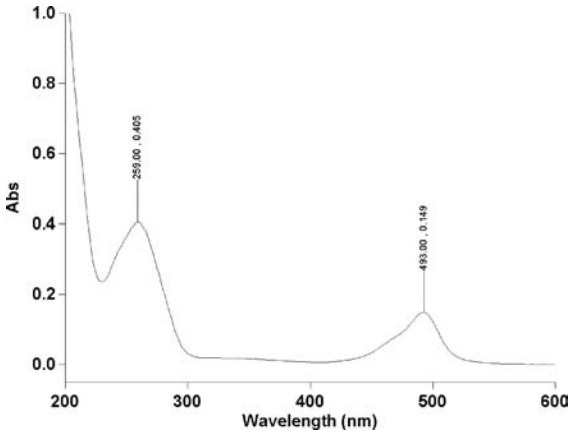

Figure 2. UV analysis of the dye-labeled DNA-22mer.

Under the same reaction condition, the corresponding nonmodified native DNA did not react with the labeling reagent (Figure S11 in Supporting Information). This indicated that the activated dye specifically reacted with the selenol (DNA-SeH), without reacting with other nucleophiles in DNA (such as the amino and phosphate groups). Analyzed by HPLC (Figure S11 in Supporting Information), efficiency of the dye incorporation into the Se-DNA is satisfactory (34% yield), comparing with literature on the fluorescent labeling (20−40% yield).[14] During the labeling, the DNA diselenide was observed as the main byproduct. The dye-labeled DNA has color of the dye (yellow) and can be purified by reversed-phase HPLC (Figure 1) or gel electrophoresis. As expected for its UV−visible spectrum (Figure 2), this dye-labeled DNA absorbs at both 260 and 490 nm, contributed by the DNA nucleobases (260 nm) and fluorescein (490 nm). In addition, their intensity ratio is approximately 3:1, which is consistent with the calculation. Analysis with MALDI-TOF mass spectrometry also confirmed the integrity of the dye-containing Se-DNA (Figure 3).

To demonstrate the usefulness of our novel strategy for DNA labeling, we performed a DNA polymerization reaction by using the fluorescein-labeled DNA as a primer. After the polymerization and gel electrophoresis, the gel was visualized by shining with blue light (450−490 nm).

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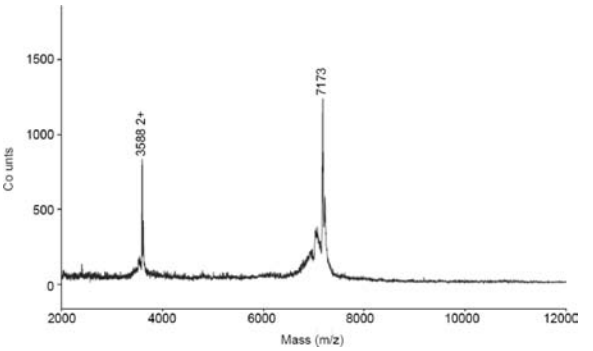

Figure 3. MS spectrum of 5′-fluorescein-labeled DNA-22mer. Its molecular formular: $C_{237}H_{284}N_{83}O_{134}P_{21}Se$; $[M]^+$: 7173 (calcd. 7169); $[M]^{2+}$: 3588 (calcd. 3585).

Because of the dye labeling, the DNA polymerization reactions became visible (Figure 4A). A time-course experiment of the polymerization reaction can be directly visualized, while the corresponding experiment using the nonlabeled DNA was not visible under blue light or UV (Figure 4B). This experiment indicated that the DNA polymerase efficiently recognized the dye-modified DNA, since the label was purposely incorporated to the 5′-terminal to avoid the potential interference with DNA polymerase. Moreover, the sensitivity of this nonradioactive labeling can reach up to the level of 30 pmol. The full-length product with the labeled dye was also confirmed by MS analysis (Figure S13 in Supporting Information). Our experimental results demonstrate that the dye-labeled Se-DNA is an efficient primer for DNA polymerase, and its reaction efficiency is virtually identical to the native DNA, since the modification site is far away from the polymerase extension site.

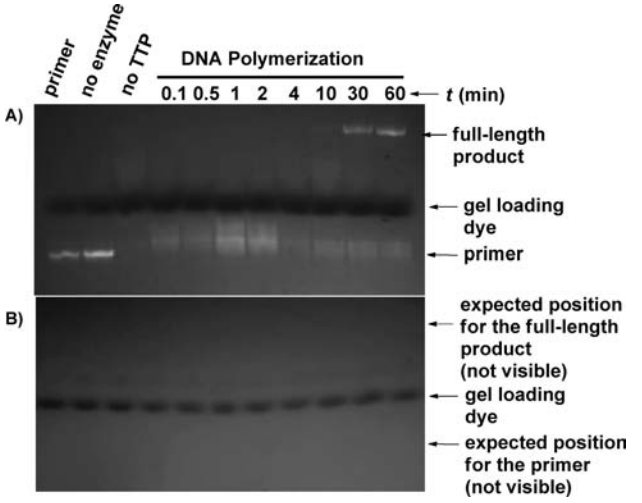

Figure 4. DNA polymerization reaction monitored with the fluorescent dye-labeled DNA-22mer. (A) Gel was shined with blue light (450−490 nm). (B) Gel was attempted to visualize by the UV shadowing.

In summary, we have successfully synthesized the 5′-Se-thymidine and the corresponding phosphoramidite and incorporated it into oligonucleotides. Using the 5′-Se-moiety for the postsynthetic modification, DNA can be simultaneously labeled under a mild condition during the deprotection step. Furthermore, we observed that the nonisotope-labeled DNA is well recognized by DNA polymerase, and DNA polymerization can be easily visualized because of the dye labeling. We have demonstrated the proof of principle of this convenient strategy for postsynthetic modification. Our facile and novel labeling strategy is useful in DNA and RNA probe preparation for convenient visualization and monitoring of bioreactions, such as DNA replication, transcription, and mRNA translation. This strategy also offers a new tool for DNA and RNA detections in disease diagnosis.

Acknowledgment. This work was financially supported by the Georgia Cancer Coalition (GCC) Distinguished Cancer Clinicians and Scientists, NIH (GM095086) and NSF (MCB-0824837).

Supporting Information Available. Experimental procedures, ^{1}H, ^{13}C and ^{31}P NMR, HRMS and MALDI-TOF MS analytical data, HPLC profiles. This material is available free of charge via the Internet at http://pubs.acs.org.