Photocleavage of a 2-nitrobenzyl linker bridging a fluorophore to the 5′ end of DNA

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Three single-stranded DNA molecules of different lengths were synthesized and characterized, each containing a fluorescent dye (6-carboxyfluorescein) connected to the 5′ end via a photocleavable 2-nitrobenzyl linker and a biotin moiety at the 3′ end. UV irradiation ($\lambda \approx 340$ nm) of solutions containing these fluorescent DNA molecules caused the complete cleavage of the nitrobenzyl linker, separating the fluorophore from the DNA. The photocleavage products were characterized by HPLC and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. Our experimental results indicated that the proximity of the chromophore 6-carboxyfluorescein to the 2-nitrobenzyl linker did not hinder the quantitative photocleavage of the linker in the DNA molecules. The biotin moiety allowed immobilization of the fluorescent DNA on streptavidin-coated glass chips. The photocleavage of the immobilized DNA was investigated directly by fluorescence spectroscopy. The results demonstrated that close to 80% of the fluorophore was removed from the immobilized DNA after UV irradiation at 340 nm. These results strongly support the application of the 2-nitrobenzyl moiety as an efficient photocleavable linker, connecting fluorescent probes to DNA molecules for a variety of biological analyses such as DNA sequencing by synthesis.

oligonucleotides | fluorescence | DNA sequencing by synthesis

The 2-nitrobenzyl group is widely used in organic synthesis as a protecting group and a cleavable linker because of its high photocleavage efficiency by near-UV light irradiation (1–4). A cleavage quantum yield of 0.49–0.63 has been reported in the literature for 1-(2-nitrophenyl)ethyl phosphate esters (5). This unique property of the photocleavable (PC) linker has promoted its application as a tool for separating, purifying, and identifying desired target biomolecules (6–8). The PC spacer phosphoramidite provides a potentially convenient tool for attachment of a wide range of functional groups to DNA by using solid-phase synthesis chemistry (8, 9).

Fluorescent oligonucleotides are unique tools to study the photophysical property of DNA (10, 11) and are widely used in biological analysis, particularly in genomics (12–14). For many of these applications, biotin is introduced site-specifically to the oligonucleotide, which can be isolated or immobilized by a streptavidin-coated solid phase (6, 15–18). In some DNA-analysis methods, it is essential to cleave off the fluorescent dye from the target molecules after detection (19, 20). For these applications as well as DNA sequencing by synthesis through fluorescence detection, it is highly desirable that the photocleavage reaction should be efficient and that side products should be minimized. We report here the synthesis, characterization, and photocleavage study of three fluorescent oligonucleotides termed 6-carboxyfluorescein (Fam)-linker-$(T)_{5,10,20}$-biotin (structure shown in Fig. 1). Each of these oligonucleotides contains a biotin moiety at the 3′ end and a fluorescent dye (Fam) connected through a PC linker (2-nitrobenzyl group) to the 5′ end. The photocleavage of the 2-nitrobenzyl PC linker in these molecules in solution and immobilized on a glass surface was studied quantitatively by HPLC and fluorescence spectroscopy. The photocleaved products were characterized by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry (MS). In the accompanying article (21), we report the design and synthesis of a fluorescent PC deoxynucleotide triphosphate for DNA labeling in a polymerase reaction. The photocleavage condition examined in this report was used to systematically study the photolysis of the polymerase reaction product.

Materials and Methods

All chemicals were purchased from Sigma–Aldrich unless otherwise indicated. Streptavidin-coated glass chips that have the capacity of binding 5 pmol of biotin per cm^2 were purchased from Xenopore (Hawthorne, NJ). Mass measurements were made on a Voyager DE MALDI-TOF mass spectrometer (Applied Biosystems). One microliter of oligonucleotide matrix solution was spotted onto a stainless-steel sample plate, and all measurements were taken in a linear positive-ion mode. The matrix solution was made by dissolving 35 mg of 3-hydroxypicolinic acid and 6 mg of ammonium citrate in 0.8 ml of 50% acetonitrile. Absorption spectra were recorded on a Lambda 40 UV/VIS spectrophotometer (Perkin–Elmer).

Synthesis of Oligonucleotides. PC fluorescent oligonucleotides Fam-linker-$(T)_{5,10,20}$-biotin were prepared by solid-phase phosphoramidite synthesis in a DNA synthesizer (Expedite 8909, Applied Biosystems) and then purified by gel electrophoresis (PAGE) and desalted with an oligonucleotide purification cartridge according to the procedure reported in literature (13). 3′-BiotinTEG-CPG, PC spacer phosphoramidite, and 5′-fluorescein phosphoramidite (Glen Research, Sterling, VA) were used to introduce biotin at the 3′ end of the oligonucleotide, and a PC 2-nitrobenzyl linker that was connected to Fam at the 5′ end. The structures of the compounds were confirmed by MALDI-TOF MS and UV-visible spectrophotometry.

Photolysis of Fam-Linker-$(T)_n$-Biotin in Solution. Fam-linker-$(T)_{5,10,20}$-biotin was dissolved in 1:1 (vol/vol) acetonitrile/water to a final concentration of 2 μM and irradiated in a quartz cell (1-cm path length) by using an LX300UV xenon lamp (ILC Technologies, Sunnyvale, CA) in conjunction with a monochromator at 340 nm (light intensity = 3 mW/cm^2). Fractions were taken out at different irradiation times for HPLC analysis.

HPLC Analysis. HPLC analysis was performed on a Waters system consisting of a Rheodyne 7725i injector, 600 Controller, Xterra MS C18 (4.6 × 50-mm) column, and 996 photodiode array detector. Elution was performed by using a linear gradient (10–25%) of methanol in a buffer that consists of 8.6 mM aqueous triethylammonium and 100 mM hexafluoroisopropyl alcohol (pH 8.1) over 60 min at a flow rate of 0.5 ml/min with

Abbreviations: PC, photocleavable; Fam, 6-carboxyfluorescein; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight.

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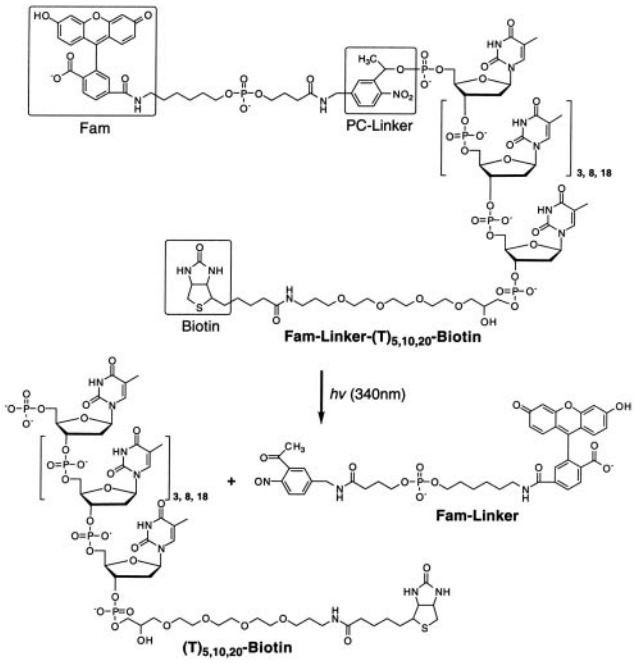

Fig. 1. Molecular structure of 5′-Fam-linker-$(T)_{5,10,20}$-biotin-3′ and their photocleavage reaction under irradiation with near-UV light.

the temperature set at 50°C. Fractions were collected and freeze-dried for MS measurement.

Photolysis of Fam-Linker-$(T)_n$-Biotin on a Glass Surface. Fam-linker-$(T)_{5,10,20}$-biotin was dissolved in 0.25 M phosphate buffer (pH 7.5) to a final concentration of 2 μM. A streptavidin-coated glass chip was immersed into the oligonucleotide solution and incubated with gentle agitation at 37°C for 5 h and then washed with water. Fluorescence emission spectra of the glass surface (λ_{ex} = 460 nm) were recorded on a Spex Fluorolog 2 1680 0.22 double spectrophotometer (Jobin-Yvon, Edison, NJ). The emitted light was collected at an angle of ≈15° from the excitation light beam. The glass chip then was put into a 2 × 2 × 7-cm chamber filled with water and irradiated with the same UV lamp at 340 nm (light intensity = 20 mW/cm^2) as described above for different periods of time. After each irradiation the glass chip was washed consecutively with water and ethanol, and then a fluorescence spectrum was recorded.

Results and Discussion

The structures of the three oligonucleotides Fam-linker-$(T)_{5,10,20}$-biotin are shown in Fig. 1. The fluorescence label Fam is connected to the 5′ end of the oligonucleotide through a PC 2-nitrobenzyl linker followed by 5, 10, and 20 thymidines (T) to produce the oligonucleotides with different chain lengths. Biotin is introduced at the 3′ end of the oligonucleotide with a triethylene glycol linker arm that allows for effective binding to a streptavidin-coated surface (22, 23). Fig. 1 shows the expected photocleavage reaction of Fam-linker-$(T)_{5,10,20}$-biotin that would produce a nitroso-derivative Fam-linker and the residual oligonucleotide $(T)_{5,10,20}$-biotin.

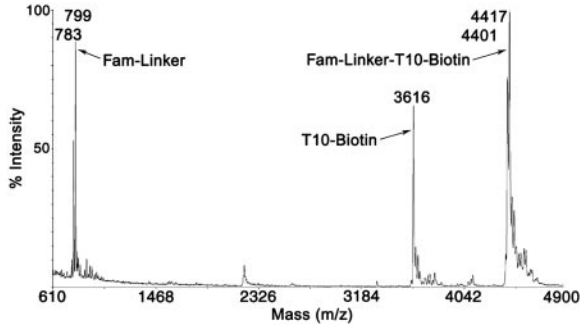

Fig. 2. MALDI-MS spectrum of Fam-linker-T_{10}-biotin: Fam-linker-T_{10}-biotin (m/z: found, 4,417; anal. calcd, 4,423), Fam-linker (m/z: found, 799; anal. calcd, 803), and T_{10}-biotin (m/z: found, 3,616; anal. calcd, 3,621).

The PC oligonucleotides were purified by PAGE and then analyzed by MALDI-TOF MS. The molecular masses of the oligonucleotides determined are presented in Table 1. The determined mass values are in good agreement with the calculated values within the accepted error margin (<9 Da) of MALDI-TOF MS measurement of DNA (24). Fig. 2 shows the mass spectrum of Fam-linker-T_{10}-biotin as a typical example. The peak at m/z 4,417 corresponds to the intact molecule Fam-linker-T_{10}-biotin, whereas peaks at m/z 3,616 and 799 are consistent with the two fragments (T_{10}-biotin and Fam-linker) from the photocleavage reaction. This result indicates that the expected photocleavage of the 2-nitrobenzyl linker (Fig. 1) occurred during the mass measurement, because MALDI MS requires irradiation of the sample by a UV laser pulse (λ = 337 nm) (25, 26). For both the signals of the intact Fam-linker-T_{10}-biotin and the cleaved Fam-linker, satellite peaks at $\Delta(m/z)$ = −16 were observed at m/z 4,401 and 783, respectively. However, the peak at m/z 3,616 corresponding to the cleaved oligonucleotide portion T_{10}-biotin did not give such a satellite peak. Thus, the satellite peaks were associated with the 2-nitrobenzyl linker. These satellite peaks were also observed for the other two fluorescent oligonucleotides Fam-linker-$T_{5,20}$-biotin, and they are assigned to the loss of an oxygen from the 2-nitrobenzyl

Table 1. Measured and calculated molecular masses (m/z) for photocleavable oligonucleotides using MALDI-TOF MS

		Calculated masses	Measured masses
Fam-linker-T_5-biotin	$(M + H)^+$; $(M - O + H)^+$	2,907; 2,891	2,900; 2,884
	$[(T_5\text{-Biotin}) + H]^+$	2,105	2,100
	$[(\text{Fam-Linker}) + H]^+$	803	799
	$[(\text{Fam-Linker}) - O + H]^+$	787	783
Fam-linker-T_{10}-biotin	$(M + H)^+$; $(M - O + H)^+$	4,423; 4,407	4,417; 4,401
	$[(T_{10}\text{-Biotin}) + H]^+$	3,621	3,616
	$[(\text{Fam-Linker}) + H]^+$	803	799
	$[(\text{Fam-Linker}) - O + H]^+$	787	783
Fam-linker-T_{20}-biotin	$(M + H)^+$; $(M - O + H)^+$	7,455; 7,439	7,457; 7,441
	$[(T_{20}\text{-Biotin}) + H]^+$	6,653	6,657
	$[(\text{Fam-Linker}) + H]^+$	803	799
	$[(\text{Fam-Linker}) - O + H]^+$	787	784

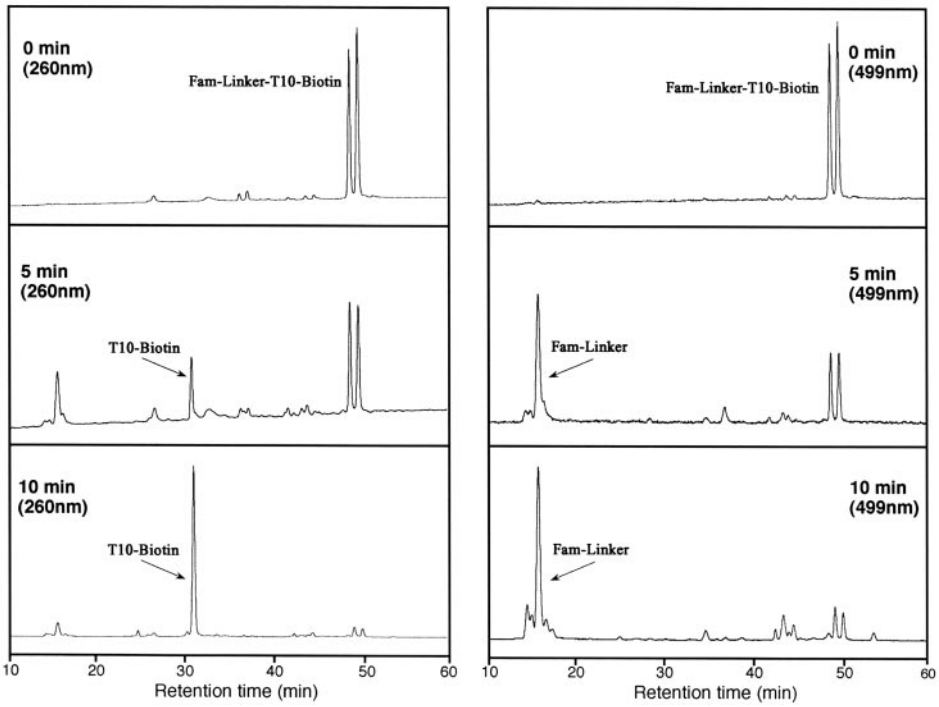

Fig. 3. HPLC analysis of the photocleavage of Fam-linker-T_{10}-biotin (2 μM) in 1:1 (vol/vol) water/acetonitrile. Detector channels: 260 nm (*Left*) and 499 nm (*Right*). (*Top*) Before irradiation; (*Middle*) after 5 min of irradiation; and (*Bottom*) after 10 min of irradiation (λ_{irr} = 340 nm).

linker moiety during the MALDI-TOF mass analysis that involves irradiation at 337 nm. This assignment is consistent with the previously reported MALDI-TOF mass analysis of an oligonucleotide containing the 2-nitrobenzyl linker introduced by PC aminotag phosphoramidite (25).

To evaluate the photocleavage reaction of the oligonucleotides in solution, we used HPLC to analyze the photocleavage products at different irradiation times. Fig. 3 shows the HPLC chromatograms of the photocleavage of Fam-linker-T_{10}-biotin at different irradiation times. Because the oligonucleotides have two specific optical absorption peaks with maxima of 260 and 499 nm corresponding to the T_{10} and Fam chromophores, respectively, detection channels at 260 and 499 nm in HPLC were used to monitor the cleavage process. Before the irradiation by UV light (time = 0 min), two peaks with absorption at both the 260- and 499-nm channel were observed at retention times of 49.0 and 49.9 min, respectively. These two peaks have experimentally indistinguishable UV-visible spectra and are attributed to the two diastereoisomeric forms of Fam-linker-T_{10}-biotin. Similar HPLC profiles of diastereoisomers of modified oligonucleotides have been reported in the literature (7, 27). Five minutes of irradiation by UV light (λ = 340 nm) resulted in a dramatic reduction in intensity of the peaks from Fam-linker-T_{10}-biotin, whereas a new peak at 15.2 min (499-nm channel) and a second new peak at 30.2 min (260-nm channel) appeared (Fig. 3). Further irradiation for 5 min more showed almost complete conversion of Fam-linker-T_{10}-biotin to its photocleavage products. The two fractions corresponding to the peaks at 15.2 and 30.2 min were collected and identified by MALDI-TOF MS as photocleavage products Fam-linker (*m/z* 795) and T_{10}-biotin (*m/z* 3618) (Fig. 4). Equivalent results were observed for the other two oligonucleotides, Fam-linker-T_5-biotin and Fam-linker-T_{20}-biotin. Thus, the oligonucleotides Fam-linker-$(T)_n$-biotin were cleaved almost completely by UV light at 340 nm in 10 min under our irradiation condition to give two photocleavage products: Fam-linker and T_n-biotin. A faster cleavage was achieved by increasing the light intensity. The combined results from Figs. 3 and 4 also indicate that the DNA moiety is stable and remains unmodified during the photocleavage reaction, which is essential for the DNA-sequencing application described in the accompanying article (21).

The efficiency of the photocleavage step depends on several factors including the efficiency of light absorption by the 2-nitrobenzyl moiety, the efficiency of the primary photochemical step, and the efficiency of the secondary thermal processes that lead to the final cleavage process (28). In the experiments described above we used a concentration of 2 μM Fam-linker-$(T)_n$-biotin. At this low concentration only a fraction of the UV

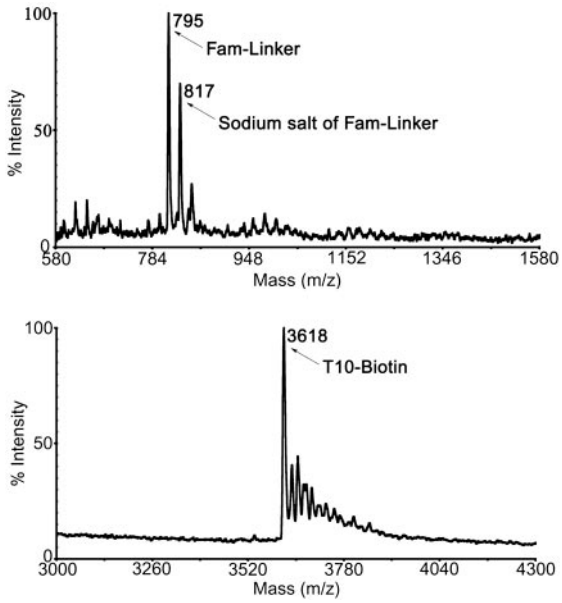

Fig. 4. MALDI-TOF MS spectra of the photocleavage products of Fam-linker-T_{10}-biotin in solution phase: Fam-linker (*m/z*: found, 795; anal. calcd, 803) and T_{10}-biotin (*m/z*: found, 3,618; anal. calcd, 3,621).

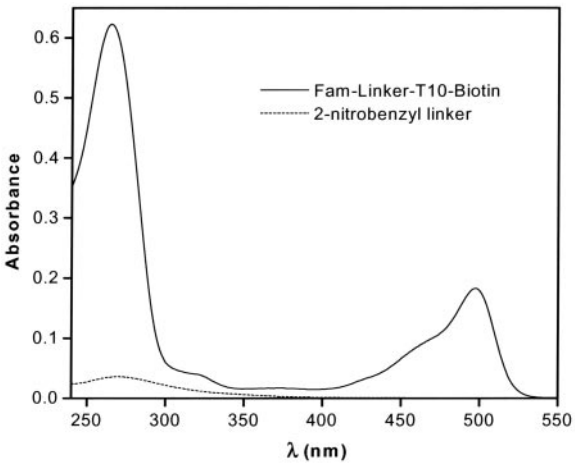

Fig. 5. Absorption spectra for Fam-linker-T_{10}-biotin and 1-[5-(aminomethyl)-2-nitrophenyl]ethanol (2-nitrobenzyl linker) with equal molar concentration (5.2 μM) obtained in 1:1 (vol/vol) water/acetonitrile.

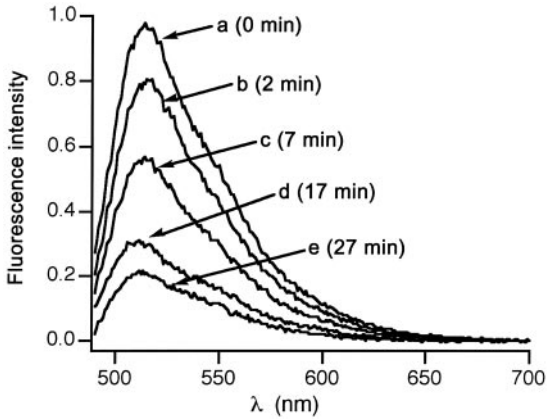

Fig. 6. Fluorescence spectra (λ_{ex} = 460 nm) of Fam-linker-T_5-biotin immobilized on a streptavidin-coated glass chip. a, before irradiation; b–e, after 2, 7, 17, and 27 min of irradiation, respectively (λ_{irr} = 340 nm, 20 mW/cm^2).

light (340 nm) was absorbed during the photocleavage process. Because there is a one-to-one relationship between the chromophore Fam and the PC linker (2-nitrobenzyl group) in Fam-linker-$(T)_n$-biotin, the ratio of absorbances of these two species will reflect the competition for light absorption at 340 nm. We calculated this value by measuring the absorption spectra (Fig. 5) of Fam-linker-T_{10}-biotin and 1-[5-(aminomethyl)-2-nitrophenyl]ethanol (2-nitrobenzyl linker) at the same molar concentration. Based on the data shown in Fig. 5, approximately half of the absorbed UV light at 340 nm is due to the absorption by the PC linker in Fam-linker-T_{10}-biotin. Our results showed that this is sufficient for quantitative photocleavage of the 2-nitrobenzyl PC linker.

For many biological applications, the oligonucleotides carrying a fluorescent dye are immobilized on a solid surface. We therefore investigated the photocleavage of the Fam-linker-$(T)_n$-biotin immobilized on a streptavidin-coated glass surface. As expected, incubation of the Fam-linker-$(T)_n$-biotin with streptavidin-coated glass chips immobilized the oligonucleotides because of the strong biotin–streptavidin interaction. Fam-linker-T_5-biotin, which has 5 thymidines, showed the highest binding capacity, whereas Fam-linker-T_{20}-biotin, which has 20 thymidines, gave the lowest binding capacity among the three PC oligonucleotides. The oligonucleotides immobilized on the glass surface were photocleaved by UV irradiation (λ = 340 nm) of the glass chip. As shown in Fig. 6, before irradiation at 340 nm, the fluorescence emission spectrum of the immobilized Fam-linker-T_5-biotin gave a strong signal (spectrum a). After 2 min of irradiation with UV light and washing thoroughly with water and ethanol, the fluorescence emission spectrum (spectrum b) of the same spot of the glass chip was taken again and showed a significant intensity decrease (80% of the original signal). Additional irradiation was applied to the same spot of the glass surface, and the fluorescence emission spectra were taken (spectra c–e). The results showed continuing decrease of the fluorescence signals (57%, 31%, and 22% of the intensity of the original signal). After further irradiation, the fluorescence signal decreased very slowly, which is probably due to the nonspecific binding of the cleaved products containing the Fam moiety trapped by the streptavidin on the glass surface. Such nonspecific binding to a streptavidin-coated surface has been observed before (29).

To confirm that the loss of Fam fluorescence of the glass chip is due to photocleavage of the fluorescent dye and not photobleaching of Fam, the following experiment was performed. Fam-linker-T_5-biotin was immobilized on a streptavidin-coated glass chip as described above and irradiated (λ = 340 nm) in dry conditions. The fluorescence emission before and after irradiation showed the same intensity of Fam fluorescence within experimental error, which confirms that Fam was not photobleached during irradiation. After the irradiation, the chip was washed with 2 ml of a water/ethanol mixture (1:1, vol/vol), and a fluorescence spectrum (λ_{ex} = 470 nm) was recorded of the resulting solution. This recording showed a strong Fam-fluorescence signal, confirming that photocleavage had occurred. The solution from the control sample without photocleavage showed no Fam-fluorescence signal. The above experiments indicate that the UV irradiation cleaves the linker without photobleaching the fluorophore.

In conclusion, the 2-nitrobenzyl linker in the oligonucleotides Fam-linker-T_n-biotin was cleaved by UV light at 340 nm in solution phase via the established mechanism with no observable side products, as was confirmed by HPLC analysis and MALDI-TOF MS. After immobilization of the oligonucleotides on a glass surface, photocleavage of the 2-nitrobenzyl linker proceeded similarly as in solution. These results provide strong support for the application of the 2-nitrobenzyl moiety as an efficient PC linker to introduce fluorescent probes into DNA molecules for a variety of biological analyses (6–8, 25). In the accompanying article (21), we used the 2-nitrobenzyl linker for the design and synthesis of a PC dye-labeled nucleotide analogue for DNA sequencing.

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