Photoreversible DNA end capping for the formation of hairpin structures†

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Received 27th November 2009, Accepted 9th February 2010
First published as an Advance Article on the web 17th February 2010
DOI: 10.1039/b924989c

We describe a photoreversible DNA end capping *via* 3-cyanovinylcarbazole nucleoside. Doubly end-capped oligodeoxynucleotide (ODN) exhibits increased stability against snake venom phosphodiesterase and shows high thermal stability.

Introduction

Hairpins[1] and pseudoknots[2] and cruciforms[3] are DNA secondary structural elements of great interest. The biological roles of these structures have been described and include regulating replication and transcription.[4] A hairpin structure is also employed in proposed biosensor and materials applications.[5] RNA too can form hairpins, playing important roles in folding and interaction with proteins.[6] Because of the stability of the hairpin structure, an intramolecular hairpin structure with various loop units has been reported. With the introduction of foreign loop molecules, intramolecular structures showed better properties such as structural stability,[7] electron transfer,[8] and therapeutic ability.[9] The crosslinking by disulfide-modified nucleic acids[10] and psoralen-derivatized nucleic acids[11] and cisplatin antitumor agents[12] have been used to stabilize hairpin structures. However, these crosslinkings cannot reversibly control the formation of hairpin structures. We have been studying artificial DNA bases as a tool for the photochemical DNA crosslinking method.[13] In our recent study, we reported that a modified oligodeoxynucleotide (ODN) containing 3-cyanovinylcarbazole nucleoside (^{CNV}K) can be photochemically crosslinked by irradiating at 366 nm with an adjacent pyrimidine base in the middle position of DNA and RNA.[14] The photocrosslinking reactions from the use of ODN containing ^{CNV}K have no limitation of sequence contexts around the photocrosslinking site. However, the photochemical reaction at the DNA strand end by using the ODN containing ^{CNV}K has not yet been investigated. Here, we report reversible photochemical end capping *via* an artificial DNA base such as ^{CNV}K at the DNA strand end. We demonstrate that doubly end-capped ODN showed stability against snake venom phosphodiesterase and high thermal stability.

Results and discussion

The phosphoramidite of ^{CNV}K was prepared according to a method reported in the literature.[14] The various modified ODNs containing ^{CNV}K were prepared, according to standard phosphoramidite chemistry, on a DNA synthesizer using the phosphoramidite of ^{CNV}K as shown in Fig. 1. ODNs containing ^{CNV}K were characterized by MALDI-TOF-MS.

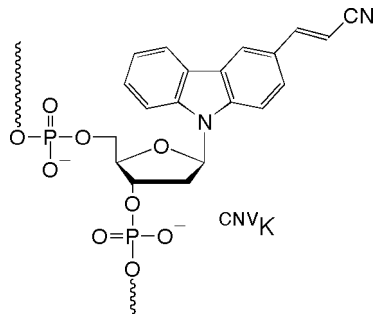

Fig. 1 Structure of 3-cyanovinylcarbazole nucleoside (^{CNV}K).

We determined the feasibility of the photochemical 5′-end capping *via* ODN containing ^{CNV}K as shown in Scheme 1. When ODN **1** (5′-d(A^{CNV}KGCGTG)-3′) and ODN **2** (5′-d(CACGCAT)-3′) were irradiated at 366 nm for 30 s, HPLC showed the appearance of a peak relating to ODN **A** in 88% yield along with the disappearance of ODN **1** and ODN **2** peaks (Fig. 2a). MALDI-TOF-MS indicates that the isolated ODN **A** obtained from HPLC purification was a photocapped product of ODN **1** and ODN **2** (calcd. 4294.97 for $[M + H]^+$; found 4294.09). The enzymatic digestion of isolated ODN **A** showed the formation of dCyd, dGuo, dThd, and dAdo in a ratio of 4 : 4 : 1 : 3, together with ^{CNV}K<>T photoadduct, which was confirmed by MALDI-TOF-MS (calcd. 599.21 for $[M + Na]^+$; found 599.21). When ODN **3** (5′-d(^{CNV}KGCGTG)-3′) and ODN **2** were used in photochemical 5′-end capping, the thymidine base reacted with photoexcited ^{CNV}K to give an end-capped product ODN **B** efficiently (Scheme 2).[15] The end capping rates by using ODN **1** were more rapid than the corresponding ODN **3**. In our previous paper,[14] the photocrosslinking between ^{CNV}K and the T base in the middle position of DNA was finished in 1 s. These results indicate that the T base of the end capping site between ODN **1** and ODN **2** was in a similar position to the T base of the photocrosslinking site. Next, we determined the feasibility of the photochemical 3′-end capping *via* ODN containing ^{CNV}K as shown in Scheme 3. When ODN **4** (5′-d(TGTGA^{CNV}K)-3′) and ODN **5** (5′-d(ATCACA)-3′) were irradiated at 366 nm for 1.0 s, HPLC showed the appearance of a peak relating to ODN **C** in 89% yield along with the disappearance of ODN **4** and ODN **5** peaks (Fig. 3a).[16] When ODN **4** and ODN **6** (5′-d(TCACA)-3′) were used in photochemical 3′-end capping, the thymidine base reacted with photoexcited ^{CNV}K to give an end-capped product ODN **D** efficiently (Scheme 4).[17] The end capping rates by using ODN **5** were almost equal to the corresponding ODN **6**. The 3′-end capping between ODN **4** and ODN containing T were finished after only 1 s.

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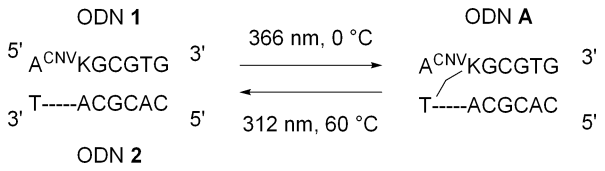
† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b924989c



Scheme 1 Photochemical 5′-end capping with ODN **1** and ODN **2**.

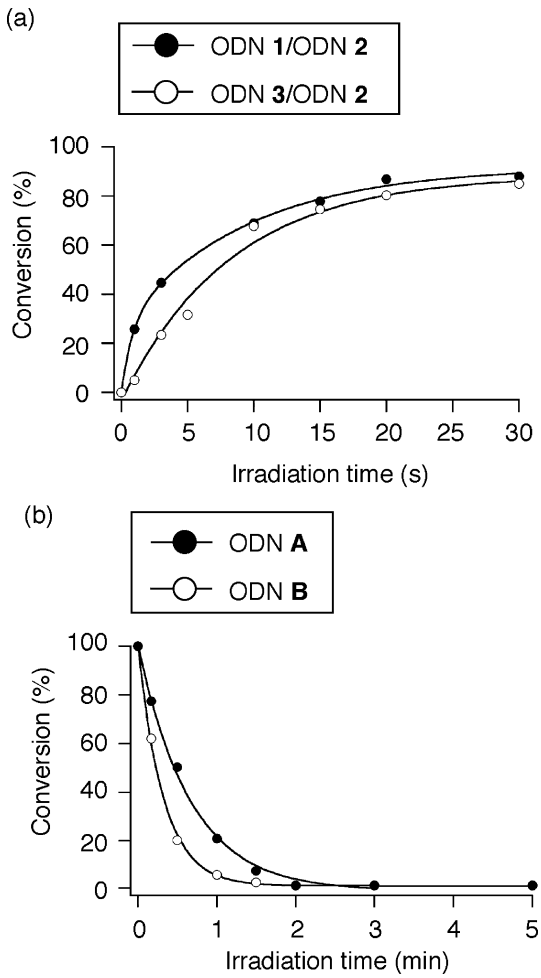
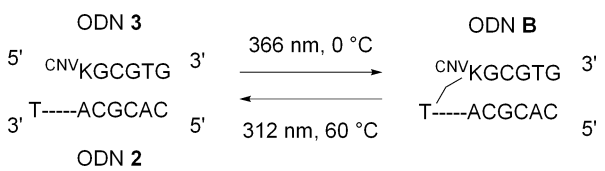
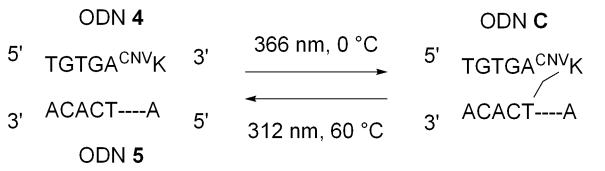


Fig. 2 (a) Time-course of photochemical 5′-end capping with ODN **1** (filled symbols) and ODN **3** (open symbols). (b) Time-course of photosplitting with ODN **A** (filled symbols) and ODN **B** (open symbols).



Scheme 2 Photochemical 5′-end capping with ODN **3** and ODN **2**.

To confirm the photoreversibility of the end-capped product, irradiation of the photocrosslinked ODN **A** at 312 nm was examined. The rapid disappearance of ODN **A** was observed by 312 nm irradiation for 2 min to revert to two ODNs (Fig. 2b), while the reverse photoreaction produced only ODN **1** and ODN **2** without any byproducts. When the photocrosslinked ODN **B** was used in the reverse photoreaction, the rapid disappearance of ODN **B** was observed by 312 nm irradiation for 1.5 min to revert to two ODNs.



Scheme 3 Photochemical 3′-end capping with ODN **4** and ODN **5**.

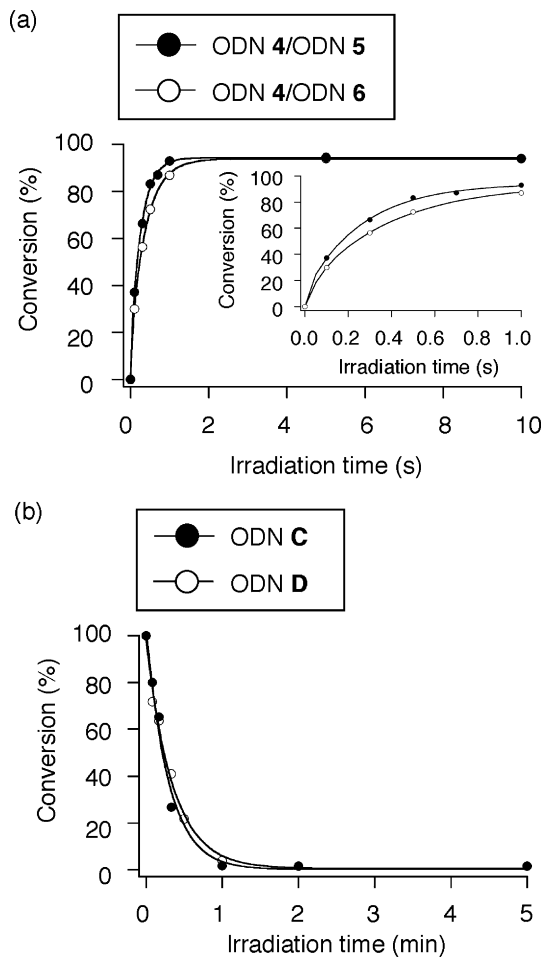
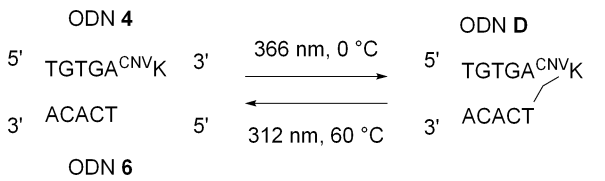


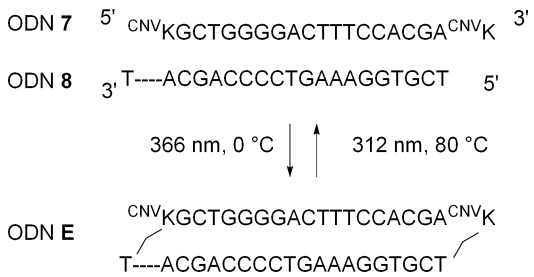
Fig. 3 (a) Time-course of photochemical 3′-end capping with ODN **5** (filled symbols) and ODN **6** (open symbols). (b) Time-course of photosplitting with ODN **C** (filled symbols) and ODN **D** (open symbols).



Scheme 4 Photochemical 3′-end capping with ODN **4** and ODN **6**.

In a manner similar to the photosplitting reaction of ODN **C** and ODN **D** the reaction proceeded rapidly to provide two ODNs (Fig. 3b). Therefore, we succeeded in the reverse reaction by irradiation at 312 nm, resulting in no damage to normal DNA. When ODN **7** (5′-d(^CNVKGCTGGGGACTTTCCACGA^CNVK)-3′) and ODN **8** (5′-d(TCGTGGAAAGTCCCCAGCAT)-3′) were irradiated at 366 nm for 40 s, the clean formation of doubly end-capped ODN **E** was observed by the densitometric assay of PAGE

(Scheme 5). Further irradiation of doubly end-capped ODN **E** at 312 nm at 80 °C resulted in a complete reversion to the original ODNs (see ESI).



Scheme 5 Photochemical doubly end capping with ODN **7** and ODN **8**.

To evaluate the stability of end-capped ODN, thermal denaturation experiments were examined (Table 1). The duplex ODN **1**/ODN **2** showed a melting temperature of 31.5 °C, whereas end-capped ODN **A** melted at 77.6 °C. An example of this behavior has been seen for end-capped ODN by the ODN-containing N^3-methyl-5-cyanovinyl-2′-deoxyuridine.[18] The duplex ODN **4**/ODN **5** showed a melting temperature of 13.3 °C, whereas end-capped ODN **C** melted at 53.6 °C. The melting temperatures of the end-capped ODNs increased more than the corresponding T4 loop hairpin ODNs. Comparing the melting temperature for the doubly end-capped ODN **E** (>85 °C) with those of the duplex ODN **7**/ODN **8** (71.8 °C) revealed an increase in thermal stability with the doubly end-capped structure.[19]

CD spectroscopy was used to study the macroscopic helical geometry of the photochemical end-capped ODN together with the duplex ODN. The duplex ODN, ODN **1**/ODN **2**, ODN **4**/ODN **5**, ODN **7**/ODN **8** displayed a characteristic B-DNA spectrum possessing a positive ellipticity at 280 nm.[20] The end-capped ODN, ODN **A**, ODN **C**, ODN **E** displayed a positive ellipticity at 280 nm in the overall CD spectrum (see ESI). Therefore, the general correspondence in the CD curves suggests that the end-capped structures adopt a B-like conformation.

We investigated the resistance of the end-capped ODN to nucleolytic digestion by snake venom phosphodiesterase (SVPD) as shown in Fig. 4.[21] After photoirradiation of the duplex ODN **7**/ODN **8** labeled with Cy3 (5′-Cy3-d(TCGTGGAAAGTCCCCAGCAT)-3′), doubly end-capped ODN **E** was used in nucleolytic digestion for 1 h compared with quantitative degradation of starting ODN **8** and the duplex ODN **7**/ODN **8**. No degradation of ODN **E** was observed after phosphodiesterase treatment for 1 h. These results show that the end-capped ODN **E** increases significantly its stability in the biological medium and its possibility as a decoy DNA for directly targeting transcription factors and for globally controlling the expression of genes.

Table 1 T_m values (°C) for duplexes[a]

ODN	T_m/°C[b]
ODN **1**/ODN **2**	31.5 ± 1.5
ODN **A**	77.6 ± 2.2
ODN **3**/ODN **2**	34.4 ± 0.9
ODN **B**	77.7 ± 1.1
ODN **9**	76.8 ± 0.6
ODN **4**/ODN **5**	13.3 ± 0.7
ODN **C**	53.6 ± 0.1
ODN **4**/ODN **6**	10.1 ± 0.8
ODN **D**	55.5 ± 0.9
ODN **10**	50.6 ± 0.1
ODN **7**/ODN **8**	71.8 ± 0.1
ODN **E**	>85

[a] All T_m values of the duplexes (3.0 μM) were measured in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM sodium chloride. ODN **9** = 5′-d(CACGCTTTTGCGTG)-3′. ODN **10** = 5′-d(TGTGTTTTCACA)-3′. [b] Each experiment was repeated at least three times.

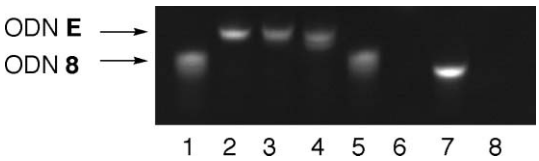


Fig. 4 16% PAGE of Cy3-labeled ODNs hydrolyzed by SVPD. Lane 1: duplex ODN **7**/ODN **8** labeled with Cy3; Lane 2: 366 nm irradiation of Lane 1 for 3 min; Lane 3: SVPD treatment of Lane 2 for 0 h; Lane 4: SVPD treatment of Lane 2 for 1 h; Lane 5: duplex ODN **7**/ODN **8** labeled with Cy3; Lane 6: SVPD treatment of Lane 5 for 1 h; Lane 7: ODN **8** labeled with Cy3; Lane 8: SVPD treatment of Lane 7 for 1 h.

Conclusions

In conclusion, we demonstrated photoreversible DNA end capping by using ODNs containing ^{CNV}K. The DNA end capping can locally stabilize the DNA strand end and can control the stability of hairpin structures by irradiation on a time-scale of seconds. Doubly end-capped ODN was more resistant to snake venom phosphodiesterase than the duplex ODNs and showed high thermal stability. Therefore, doubly end-capped ODN can be proposed as a powerful decoy DNA to the inhibition of gene expression.

Experimental

General

Mass spectra were recorded on a Voyager-DE PRO-SF, Applied Biosystems. Irradiation was performed by UV-LED (OMRON, ZUV, 366 nm, 1,600 mW cm^{-2}) or 15 W transilluminator (FU-NAKOSHI, TR-312R/J, 312 nm). HPLC was performed on a Chemcobond 5-ODS-H column (10 × 150 mm, 4.6 × 150 mm) or a Chemcosorb 5-ODS-H column (4.6 × 150 mm) with a JASCO PU-980, HG-980-31, DG-980-50 system equipped with a JASCO UV 970 detector at 260 nm. The reagents for the DNA synthesizer such as A, G, C, T-β-cyanoethyl phosphoramidite, and CPG support were purchased from Glen Research. Phosphodiesterase I was purchased from USB Corporation.

Preparation of ODN

ODN sequences were synthesized by the conventional phosphoramidite method by using an Applied Biosystems 3400 DNA synthesizer. The coupling efficiency was monitored with a trityl monitor. The coupling efficiency of a crude mixture of ^{CNV}K was 97% yield. The coupling time of a crude mixture of ^{CNV}K was 999 s. They were deprotected by incubation with 28% ammonia for 4 h at 65 °C and were purified on a Chemcobond 5-ODS-H column

(10 × 150 mm) by reverse phase HPLC; elution was with 0.05 M ammonium formate containing 3–25% CH_3CN, linear gradient (30 min) at a flow rate of 2.5 mL min^{-1}. Preparation of ODNs was confirmed by MALDI-TOF-MS analysis. MALDI-TOF-MS: calcd 2229.58 for ODN **1** [$(M + H)^+$], found 2229.23; calcd 1916.37 for ODN **2** [$(M + H)^+$], found 1916.98; calcd 1915.38 for ODN **4** [$(M + H)^+$], found 1915.91; calcd 6309.25 for ODN **7** [$(M + H)^+$], found 6309.06.

Photochemical end capping of ODNs as monitored by HPLC

The reaction mixture (total volume 120 μL) containing ODN **1** and ODN **2** (each 20 μM, strand concn.) in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM sodium chloride was irradiated with a UV-LED (366 ± 15 nm light) at a distance of 1.5 cm at 0 °C. After irradiation, the progress of the photoreaction was monitored by HPLC. The yield was calculated on the basis of ODN **2**.

Photosplitting of ODNs as monitored by HPLC

A solution (total volume 60 μL) containing ODN **A** (20 μM, strand concn.) in H_2O was irradiated with 15 W transilluminator (312 nm) at 60 °C. After irradiation, the progress of the photoreaction was monitored by HPLC. The yield was calculated on the basis of ODN **A**.

Spectroscopic measurements

UV spectra of DNA (3.0 μM) were taken in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM sodium chloride using a JASCO V-550 UV-VIS spectrophotometer or a Beckman Coulter DU800 UV/VIS spectrophotometer. In T_m measurements of the duplex, sigmoidal curves on the change of A_{260} were obtained, and the T_m value was calculated from the first part of the curve. The CD spectra were measured from 200 to 350 nm in a 0.1 cm path length cuvette. All spectra of the duplexes (30 μM, strand concn.) were measured at 10 °C in a buffer containing 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM sodium chloride. CD spectra were recorded on a JASCO J-820 spectrometer.

Stability of the end-capped product with snake venom phosphodiesterase

To a solution (10 μL) containing ODN **E** labeled with Cy3 (2 μM, strand concn.) or ODN **8** labeled with Cy3 (2 μM, strand concn.), snake venom phosphodiesterase (10 μL, 6 mUnits) was added and incubated at 37 °C. After the reaction mixture was heated at 95 °C for 5 min, the reaction mixture was analyzed by electrophoresis on 16% polyacrylamide gel containing 8 M urea. The labeled ODN in the gel was visualized by a LAS-3000 system (Fujifilm).

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas by the Ministry of Education, Science, Sports and Culture of Japan and by a grant from the New Energy and Industrial Technology Development Organization (NEDO).

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16 MALDI-TOF-MS: calcd 3675.59 for ODN **C** [$(M + H)^+$], found 3675.54; calcd 599.21 for $^{CNV}K<>T$ photoadduct [$(M + Na)^+$], found 599.20. The yield was calculated on the basis of ODN **5**.
17 MALDI-TOF-MS: calcd 3362.39 for ODN **D** [$(M + H)^+$], found 3362.29; calcd 599.21 for $^{CNV}K<>T$ photoadduct [$(M + Na)^+$], found 599.19. The yield was calculated on the basis of ODN **6**.
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