Photo-Cross-Linkable Artificial Nucleic Acid: Synthesis and Properties of 3-Cyanovinylcarbazole-Modified Nucleic Acids and Its Photo-Induced Gene-Silencing Activity in Cells

Takashi Sakamoto and Kenzo Fujimoto

Abstract The inter-strand photo-cross-linking reaction between nucleic acid strands has wide potential for regulating gene expression specifically and spatiotemporally due to its sequence specificity and high photo irradiation operability. Therefore, photo-cross-linkable artificial nucleic acids are required to be specific and effective drugs without adverse effects and also be good tools for investigating gene functions in cells.

As one of the most reactive photo-cross-linkable artificial nucleic acids, in this review, 3-cyanovinylcarbazole modified oligodeoxyribonucleotides that can photocrosslink with their complementary nucleic acid within a few seconds of photoirradiation are examined. The details of the synthetic method, properties and the applications for regulating gene expression in cells are discussed.

Keywords Inter-strand photo-cross-linking · Nucleic acids · Photoirradiation · 3-cyanovinylcarbazole · Gene expression · Antisense oligonucleotides

1 Introduction

Inter-strand photo-cross-linking of nucleic acids is one of the most useful technologies for regulating nucleic acid functions, such as transcription and replication of the genome, gene silencing activity of microRNAs, and translational activity of mRNAs. The sequence specific and thermally irreversible covalent bond formation between the nucleic acid strand and artificial photo-cross-linkable oligonucelotide caused by the photo-cross-linking reaction enables us to regulate sequence-specifically and spatiotemporally the target nucleic acid function.

T. Sakamoto · K. Fujimoto (✉)
School of Materials Science, Japan Advanced Institute of Science and Technology, Nomi, Ishikawa, Japan
e-mail: kenzo@jaist.ac.jp

S. Obika, M. Sekine (eds.), *Synthesis of Therapeutic Oligonucleotides*,
https://doi.org/10.1007/978-981-13-1912-9_10

As the pioneering works in this field, in the late 1980s, Miller and co-workers reported psoralen-modified oligonucleotide (Fig. 1, left) as the first photo-cross-linkable synthetic oligonucleotide [1, 15, 24]. They introduced trioxalen, a natural DNA photo-cross-linker that can be obtained from plants, at the 5′ end of synthetic oligodeoxyribonucleotides (ODN(s)), and clearly demonstrated that the ODNs cross-linked with single-stranded DNA and double-stranded DNA [14] under UVA (365 nm) irradiation. The findings led to the study on photoresponsive synthetic oligonucleotides. In the late 2000s, advanced-type ODNs having psoralen-modified adenosine were reported by Murakami and co-workers (Fig. 1, center) [11, 13]. Due to the restricted and favorable position of psoralen moiety in double-stranded nucleic acid consisting of the ODN and the complementary nucleic acid, these ODNs have higher photoreactivity compared to 5′ psoralen-modified ODNs. The psoralen-modified ODNs can photo-cross-link with not only single-stranded DNA, but also with single-stranded RNA and double-stranded DNA. The wide photoreactivity toward nucleic acid strands enables us to photoregulate various nucleic acid functions, such as transcription, [2, 7, 8, 18, 19, 29, 31] translation [3, 16] and microRNA activity [17].

In our group, as the next generation of photo-cross-linkers, 3-cyanovinylcarbazole-modified oligonucleotides (Fig. 2) have been developed [26, 30]. Since it has extremely high photoreactivity compared to other artificial photo-cross-linkable ODNs, the 3-cyanovinylcarbazole-modified ODNs can photo-cross-link with complementary DNA or RNA within a few seconds of UVA irradiation. Currently, the 3-cyanovinylcarbazole-modified photo-cross-linkable ODNs are applied to various fields of scientific study, such as the construction of DNA-based

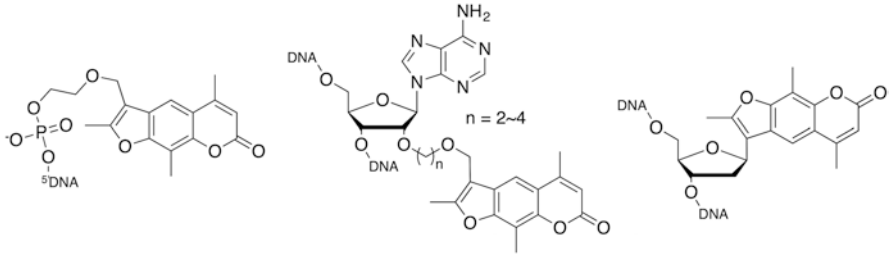


Fig. 1 Psoralen-modified photo-cross-linkable oligonucleotides

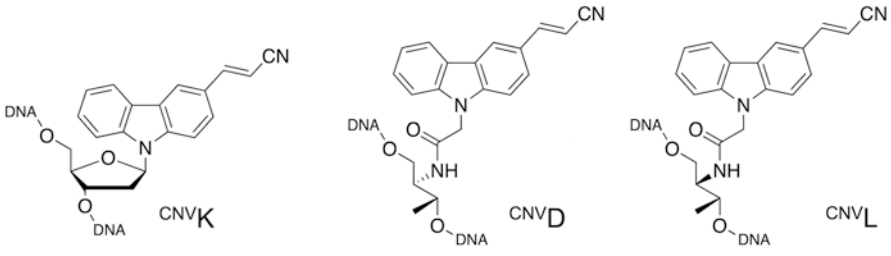


Fig. 2 3-cyanovinylcarbazole-modified photo-cross-linkable oligonucleotides

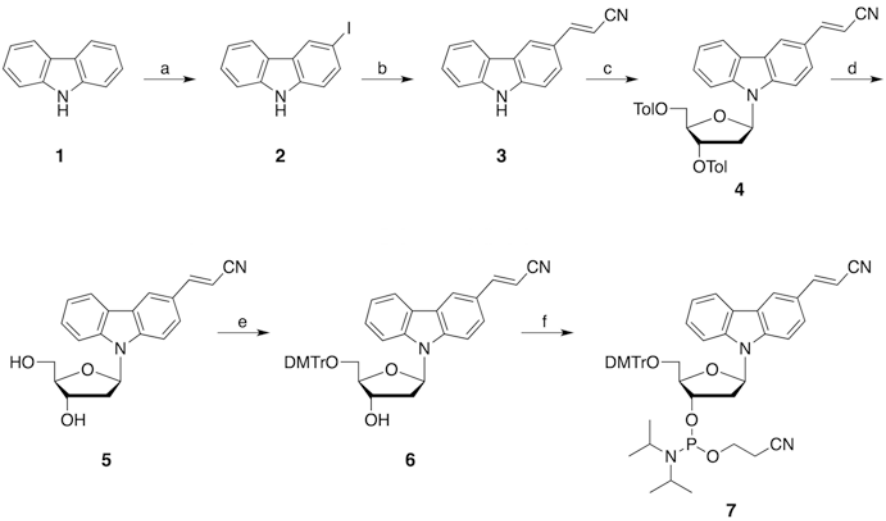
nanomaterials [23], SELEX-based aptamer selection [21], site-specific nucleic acid editing [4, 5], and gene regulation in cells [25].

In this chapter, the synthetic methods and properties of the 3-cyanovinylcarbazole-modified ODNs, and the applications for gene silencing are described.

2 Synthesis of 3-Cyanovinylcarbazole-Based Photo-Cross-Linker

Currently, 3-cyanovinylcarbazole-modified ODN is the most reactive photo-cross-linkable ODN in the world. However, at this time, the reagents for the modification of ODNs are not commercially available. Therefore, in this section, a practical method to synthesize the 3-cyanovinylcarbazole-modified ODNs is described.

The phosphoramidite monomer of ^{CNV}K can be obtained by multi-step organic synthesis as shown in Scheme 1 from commercially available carbazole (**1**) as a starting material. At first, the electron rich C3 carbon of carbazole is iodized by a general electrophilic halogenation procedure using $NaIO_4$ as a catalyst. In this reaction, 3,6-diiodocarbazole by-product is sometimes generated because the electron density of C6 carbon of the target product, e.g. 3-iodocarbazole (**2**), is larger than that of the starting material. To obtain the target within a high yield, the homogeneity and the molar equivalent of all the reagents should be carefully checked before starting the reaction. 3-Cyanovinylcarbazole (**3**) can be obtained easily by the

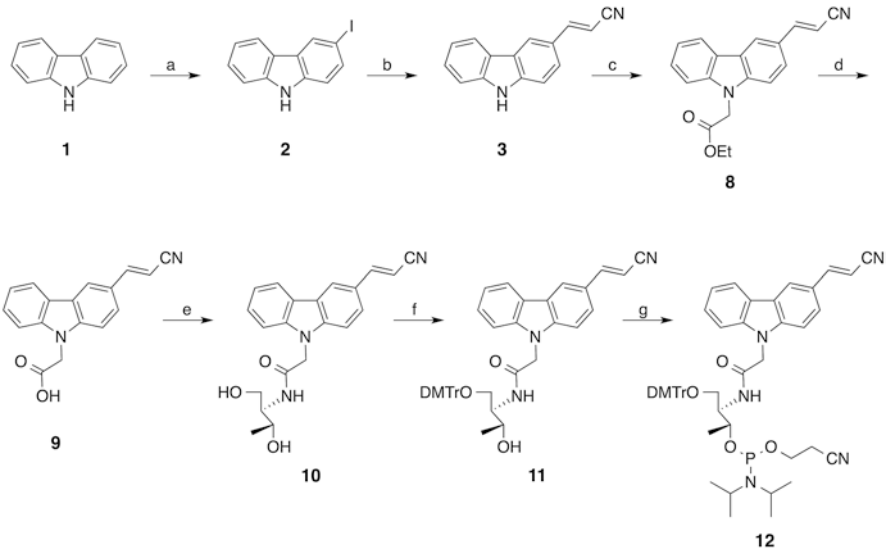


Scheme 1 Synthesis of the phosphoramidite monomer of ^{CNV}K. a) I_2, $NaIO_4$, H_2SO_4, EtOH; b) acrylonitrile, $Pd(OAc)_2$, tributylamine, DMF-H_2O;c) KOH, TDA-1, chlorosugar, MeCN; d) MeONa, MeOH, $CHCl_3$; e) DMTr-Cl, DMAP, Pyridine; g) $(iPr_2N)_2POCE$, Benzylthio-1H-tetrazole, MeCN

Mizoroki-Heck reaction [10, 20] using acrylonitrile and $Pd(OAc)_2$ as a vinyl donor and palladium catalyst, respectively. In this reaction, a microwave synthesizer can be used for quick and high-yield preparation of the target compound. By adopting this equipment, the reaction is finished within 10 min. To couple 3-cyanovinylcarbazole (**3**) and the deoxyribose ring, 1-chloro-2-deoxy-3,5-di-O-p-toluoyl-D-ribofuranose (chlorosugar) can be used as a commercially-available reagent. The chlorosugar can also be obtained by the literature method [9]. Briefly, the methoxy substitution of 1 OH of 2-deoxy-D-ribofuranose is performed with methanol solution of acetyl chloride, the toluoyl protection of 5 and 3 OH groups of 1′-methoxy-2′-deoxy-D-ribofuranose is carried out using p-toluoyl chloride, and then the chlorination of 1 carbon of 1-methoxy-2-deoxy-3,5-di-O-p-toluoyl-D-ribofuranose is performed with HCl generated from acetyl chloride. According to this procedure, chlorosugar can be obtained as white precipitate generated in the reaction mixture; therefore, we can obtain chlorosugar just by using a simple filtration procedure.

After the deprotection of the p-toluoyl group at 5′ and 3′ OH groups of protected 3-cyanovinylcarbazole-1′-β-deoxyriboside using sodium methoxide, 3-cyanovinylcarbazole-1′-β-deoxyriboside (**5**, ^{CNV}K) can be obtained in good yield. Phosphoramidite monomer of ^{CNV}K can be obtained by 5′ OH DMTr protection and 3′ OH phosphoramidite modification with general procedures, respectively.

The phosphoramidite monomer of ^{CNV}D can be obtained by a multi-step organic synthesis (Scheme 2) from commercially available carbazole (**1**). After the synthesis of 3-cyanovinylcarbazole (**3**) using the same procedures of ^{CNV}K synthesis, an acetic acid derivative of 3-cyanovinylcarbazole, 3-cyanovinylcarbazol-9-yl-acetic



Scheme 2 Synthesis of the phosphoramidite monomer of ^{CNV}D. a) I_2, $NaIO_4$, H_2SO_4, EtOH; b) acrylonitrile, $Pd(OAc)_2$, tributylamine, DMF-H_2O; c) K_2CO_3, $BrCH_2CO_2Et$; d) HCl; e) D-threoninol, WSC, HOBt, DMF; f) DMTr-Cl, DMAP, Pyridine; g) $(iPr_2N)_2POCE$, Benzylthio-1H-tetrazole, MeCN

acid (**5**), can be obtained easily by the coupling with ethyl bromoacetate, and hydrolysis of the ethyl ester. *N*-(3-cyanovinylcarbazol-9-yl-acetyl) D-threoninol (**10**, ^{CNV}D) can be obtained by a simple condensation reaction with carbodiimide. Phosphoramidite monomer of ^{CNV}D can be obtained by DMTr protection of primary alcohol and phosphoramidite modification of secondary alcohol with general procedures, respectively. ^{CNV}L can also be obtained with the same synthetic procedures using L-threoninol instead of D-threoninol in the case of ^{CNV}D.

2.1 Synthetic Procedures of ^{CNV}K and Its Phosphoramidite Monomer

2.1.1 3-Iodocarbazole (2)

To a solution of carbazole (8.0 g, 47 mmol), iodine (6.0 g, 24 mmol) and $NaIO_4$ (2.5 g, 12 mmol) in EtOH (400 mL) was added a solution of H_2SO_4 (5 mL) in EtOH (200 mL), and stirred at 65 °C for 1 h. After neutralization with NaOH, solvent was evaporated, redissolved in CH_3Cl (700 mL) and washed with H_2O and brine. The organic layer was condensed and recrystallized twice to give **2** as a white solid (4.9 g, 17 mmol, 36%).

2.1.2 3-Cyanovinylcarbazole (3)

To a suspension of **2** (1.5 g, 5.12 mmol) in 20% DMF/H_2O (2.8 mL) in a pressure-resistant glass tube, palladium acetate (115 mg, 0.51 mmol), tributylamine (1.22 mL, 5.12 mmol) and acrylonitrile (0.84 mL, 12.8 mmol) were added, and microwave irradiated (60 W, 160 °C) by a microwave synthesizer (DISCOVER, CEM) for 10 min. The reaction was monitored by TLC (hexane/AcOEt, 4:1), which showed the absence of the starting material. After the reaction mixture was evaporated, the residue was chromatographed on a silica gel using hexane/AcOEt (3:1, v/v) as eluent to give **3** (0.85 g, 76%) as a white powder.

2.1.3 3-Cyanovinylcarbazole-9-yl-1′-β-deoxyriboside-3′,5′-di-(P-toluoyl) ester (4)

To a solution of potassium hydroxide (2.29 g, 40.9 mmol) and tris[2-(2-methoxyethoxy)ethyl]amine (90.0 mg, 0.28 mmol) in CH_3CN (360 mL), **3** (3.02 g, 13.9 mmol) was added and then stirred at room temperature for 30 min. To this reaction mixture chlorosugar (6.09 g, 14.3 mmol) was added and then stirred at room temperature for 60 min. The reaction was monitored by TLC (hexane/AcOEt, 4:1), which showed the absence of the starting material. After the reaction mixture was evaporated, the residue was chromatographed on a silica gel using $CHCl_3$ as eluent to give **4** (7.89 g, 99%) as a yellow powder.

2.1.4 3-Cyanovinylcarbazole-9-yl-1′-β-deoxyriboside (5)

To a solution of **4** (7.89 g, 13.8 mmol) in MeOH (400 mL) was added 0.5 M methanolic MeONa (83.0 mL, 41.5 mmol) and $CHCl_3$ (50 mL) and then stirred at room temperature for 1 h. The reaction was monitored by TLC ($CHCl_3$/MeOH, 9:1), which showed the absence of starting material. After the reaction mixture was evaporated, the residue was chromatographed on a silica gel using $CHCl_3$/MeOH (9:1, v/v) as eluent to give **5** (2.93 g, 63%) as a white powder.

2.1.5 5′-O-(4,4′-Dimethoxytrityl)-3-cyanovinylcarbazole-9-yl-1′-β -deoxyriboside (6)

To a solution of **5** (2.83 g, 8.47 mmol) in pyridine (12 mL) was added a solution of 4,4′-dimethoxytrityl chloride (3.45 g, 10.2 mmol) and 4-(dimethylamino)pyridine (0.21 g, 1.70 mmol) in pyridine (24 mL) on an ice bath and then stirred at room temperature for 23 h. After the reaction mixture was evaporated, the residue was chromatographed on a silica gel using $CHCl_3$/MeOH (99:1, v/v) as the eluent to give **6** (4.20 g, 78%) as yellow foam.

2.1.6 5′-O-(4,4′-Dimethoxytrityl)-3-cyanovinylcarbazole-9-yl-1′-β -deoxyriboside-3′-O-(cyanoethoxy-*N*,*N*-diisopropylamino) phosphoramidite (7)

6 (0.32 g, 0.50 mmol) in a rubber sealed bottle was dissolved in CH_3CN (1 mL) and co-evaporated three times in vacuo. A solution of 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphoramidite (160 μL, 0.50 mmol) in CH_3CN (2.2 mL) and 0.45 M *1H*-tetrazole in CH_3CN (1.18 mL, 0.50 mmol) were added and then stirred at room temperature for 2 h. The reaction mixture was diluted with AcOEt (15 mL) and washed with a saturated aqueous solution of $NaHCO_3$, and water. The organic layer was collected, dried over anhydrous magnesium sulfate, filtered, and evaporated in vaccuo to give the crude product of **7** (0.42 g, quant.) and was used as the automated DNA synthesizer without further purification.

2.2 Synthetic Procedures of ^{CNV}D and Its Phosphoramidite Monomer

2.2.1 Ethyl 3-cyanovinylcarbazol-9-yl-acetate (8)

3-Cyanovinylcarbazole (**3**) (1.1 g, 5 mmol) and NaH (60% oil suspension, 0.24 g, 6 mmol) were dissolved in dry DMF (20 mL) and stirred for 1 h at room temperature under N_2 atmosphere. Ethyl bromoacetate (1.1 mL, 9.9 mmol) was added

drop-wisely over 30 min. The reaction mixture was diluted with water (300 mL) and extracted with chloroform and then dried over sodium sulfate. After removal of the solvent, the residue was subjected to silica gel column chromatography (0–1% MeOH/$CHCl_3$) to yield **4** as white solid (1.4 g, 4.6 mmol, 92%).

2.2.2 3-Cyanovinylcarbazol-9-yl-acetic Acid (9)

4 (1.4 g, 4.6 mmol) and NaOH (0.18 g, 23 mmol) were dissolved in THF/MeOH/H_2O (3:2:1, 30 mL) and stirred for 2 h at ambient temperature. After the addition of 1 N HCl (250 mL), the reaction mixture was extracted with EtOAc (300 mL) and washed with 1 N HCl. The organic layer was dried over $MgSO_4$ and evaporated to yield **5** as white solid (1.2 g, 4.4 mmol, 96%).

2.2.3 *N*-(3-Cyanovinylcarbazol-9-yl-acetyl)-D-threoninol (10, ^{CNV}D)

5 (1.3 g, 4.5 mmol) and D-threoninol (0.47 g, 4.5 mmol) were added to dry DMF (20 mL) containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; 0.86 g, 4.5 mmol) and 1-hydroxybenzotriazole (HOBt; 0.69 g, 4.5 mmol), and stirred for 5 h at ambient temperature. Sat. NaCl aq. soln. was added and the obtained precipitate was collected and dried under vacuo to yield **6** as white solid (1.5 g, 4.1 mmol, 91%).

2.2.4 *N*-(3-Cyanovinylcarbazol-9-yl-acetyl)-1′-*O*-(4,4′-dimethoxytrityl)-D-threoninol (11)

A solution of **6** (0.40 g, 1.1 mmol), 4,4′-dimethoxytritylchloride (0.41 g, 1.2 mmol) and 4-dimethyl- aminopyridine (DMAP; 30 mg, 0.25 mmol) in dry pyridine (2 mL) were stirred at ambient temperature for 20 h. The reaction mixture was diluted with $CHCl_3$, washed with H_2O and the organic layer was dried over $NaSO_4$. After the removal of the solvent, residue was subjected to silica gel column chromatography ($CHCl_3$ with 0.2% TEA) to yield **7** (0.63 g, 86%).

2.2.5 *N*-(3-Cyanovinylcarbazol-9-yl-acetyl)-1′-*O*-(4,4′-dimethoxytrityl)-D-threoninol 3′-*O*-(Cyanoethoxy-*N,N*-diisopropylamino) phosphoramidite (12)

The residual trivial amount of water in **7** was removed by azeotropic distillation with dry acetonitrile (twice). Then, **7** (0.38 g, 0.57 mmol) was dissolved with 0.25 M solution of 5-benzylthio-*1H*-tetrazole in dry MeCN (2.3 mL, 0.57 mmol) and a solution of 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphoramidite (0.18 mL, 0.57 mmol) in dry acetonitrile (5 mL) was added and stirred under N_2 atmosphere

for 2 h. The crude mixture was dissolved in ethyl acetate. The solution containing **8** was washed with water, sat. $NaHCO_3$ aq. soln. and brine. The organic layer was dried over $MgSO_4$, and then filtered and the ethyl acetate was removed. The yellow foam (0.48 g, 0.55 mmol, 96%) was obtained and immediately used for DNA synthesis without further purification.

2.3 Synthesis of the Oligonucleotide Having ^{CNV}K or ^{CNV}D

ODNs having ^{CNV}K or ^{CNV}D can be obtained by the conventional cyanoethylphosphoramidite chemistry using phosphoramidite monomer of ^{CNV}K or ^{CNV}D. If the automated DNA synthesizer has the extra phosphoramidite ports, ODNs having ^{CNV}K or ^{CNV}D can be synthesized easily by an automated DNA synthesizer. Due to the high stability of 3-cyanovinylcarbazole in cleavage and deprotection conditions, the general conditions (28% ammonium hydroxide, 55 °C, 8 h) are applicable. Crude ODN solution can be purified by reversed-phase HPLC in the same manner as general ODN purification.

2.4 Further Modification of ^{CNV}K- or ^{CNV}D-Modified ODNs

As the OH group on the 5′ and 3′ termini of the ^{CNV}K- or ^{CNV}D-modified ODNs is free for further modification, the general phosphoramidite-based modification method using 5′ or 3′ modifiers, such as FAM, biotin, amino, thiol, on the automated DNA synthesis is available. In the case of fluorescence labeling of the ODNs, a fluorophore whose stability is sufficiently high during the photo-cross-linking reaction, should be selected. In our cases, the Cy3 and Cy5 label is frequently used as the photo-stable fluorophore. To the best of our knowledge, fluorescein is not suitable for the fluorescent label of ^{CNV}K or ^{CNV}D-modified ODNs because of its lower photostability under UV irradiation.

3 Inter-Strand Photo-Cross-Linking Using ODNs Having ^{CNV}K or ^{CNV}D

In this section, the properties of the photo-cross-linking reaction of ^{CNV}K and ^{CNV}D, which currently are the most highly reactive photo-cross-linkers in the world, are described. Basic findings described here will enable the advanced application of the photo-cross-linking reaction of these photo-cross-linkable ODNs having ^{CNV}K or ^{CNV}D.

3.1 *Properties of the Inter-Strand Photo-Cross-Linking Reaction of ^{CNV}K and ^{CNV}D in Nucleic Acid Double Strands*

The inter-strand photo-cross-linking reaction of ^{CNV}K and ^{CNV}D occurs between the vinyl group of 3-cyanovinylcarbazole moiety and the C5-C6 double bond of the pyrimidine base situated at the –1 position of ^{CNV}K or ^{CNV}D in the complementary DNA or RNA strand with 366 nm UV irradiation (Fig. 3). Since the 312 nm irradiation under the denaturing condition causes reverse photoreaction and photo-splitting, the stability of the hybrid can be regulated freely by UV irradiation. As shown in Fig. 4, the local sequence around ^{CNV}K or ^{CNV}D largely affects the yield of the photo-cross-linking reaction. This indicates that the local duplex structure is important for the photo-cross-linking reaction. These reactions, photo-cross-linking and photo-splitting, can be monitored quantitatively by HPLC or denaturing PAGE analysis as the change in the retention time or mobility, respectively.

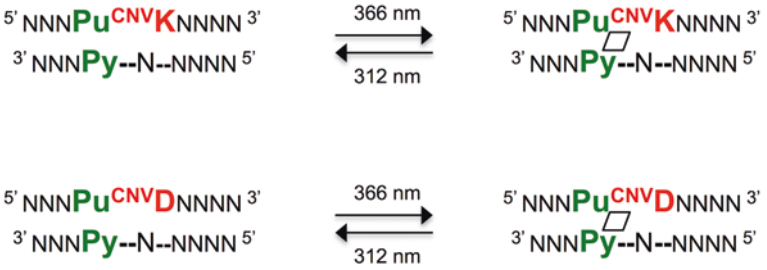


Fig. 3 Schematic drawing of the photo-cross-linking and photo-splitting reaction of 3-cyanovinylcarbazole-modified ODNs

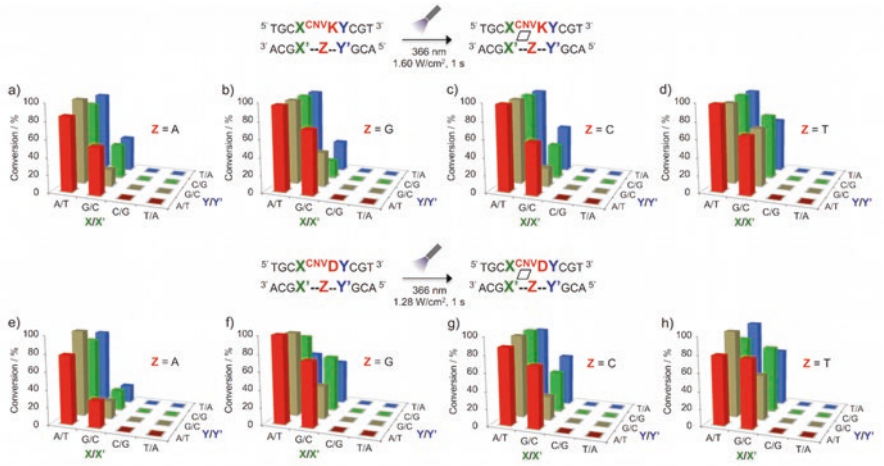


Fig. 4 Sequence dependency of the photo-cross-linking yields of ^{CNV}K- or ^{CNV}D-modified ODNs

^{CNV}K, which was first reported in 2008 [30], has great photoreactivity in the interstrand photo-cross-linking reaction in the DNA double-strand. The reaction is finished within a few seconds of 366 nm irradiation using UV-LED. Although the pyrimidine bases, T and C, in the complementary strand can be cross-linked with ^{CNV}K, the reactivity depends on the bases, and the reactivity toward C is 27-fold lower than that toward T. This large difference of the photoreactivity dependent on the target pyrimidine base sometimes restricts the design of the ODN sequence for the photoreaction. In 2014, ^{CNV}D, which has a more flexible skeleton compared to ^{CNV}K, was developed [26] as the improved version of ^{CNV}K. The photoreactivity of ^{CNV}D is ca. 1.8-fold (toward T) and eightfold (toward C) greater than that of ^{CNV}K. In the case of ^{CNV}D, since the difference of the photoreactivity between T and C is closer than that of ^{CNV}K, the freedom for designing the sequences for the photo-cross-linking reaction is increased.

3.2 Light Source

For the photoreaction of ^{CNV}K and ^{CNV}D, a high-power UV-LED and a general transilluminator can be used depending on the experimental requirements. As the irradiation spotlight of UV-LED is small, ca. 100 mm^2 at maximum, this light source is not suitable for the simultaneous irradiation of one lot of samples. If the experiment requires simultaneous irradiation of the samples, the transilluminator is suitable because the area that can be uniformly irradiated is larger, ca. 40,000 mm^2, than that of UV-LED. In the case of the use of the transilluminator, the photoreaction proceeds more slowly compared to the case of UV-LED because of its lower irradiation energy compared to UV-LED.

3.3 Structural Insight of the DNA Duplex Including ^{CNV}K

As shown in Fig. 5, it was demonstrated that only the trans isomer of ^{CNV}K can react with the pyrimidine base on the complementary strand, and that reaction gives the photoadduct that was formed from the trans isomer of ^{CNV}K [6]. As the rate of the photo-isomerization of the cyanovinyl group is higher than that of the [2+2] photocycloaddition reaction, the rate determining step of the photo-cross-linking is the [2+2] photocycloaddition reaction. According to the structure of the photoadduct, the duplex structures consisting of ^{CNV}K-modified ODN and its complementary ODN before and after the photo-cross-linking reaction can be estimated by energy minimization calculation. As shown in Fig. 6, the structure of the duplex after the photo-cross-linking is slightly bent compared to that of before the photo-cross-linking. From the distance between the carbon atoms on certain nucleoside units, the major axis of the duplex is bent ca. 20° by the photo-cross-linking reaction. The degree of bend is comparable to the result that measured the bend in the ^{CNV}K photo-cross-linked DNA tile array [28].

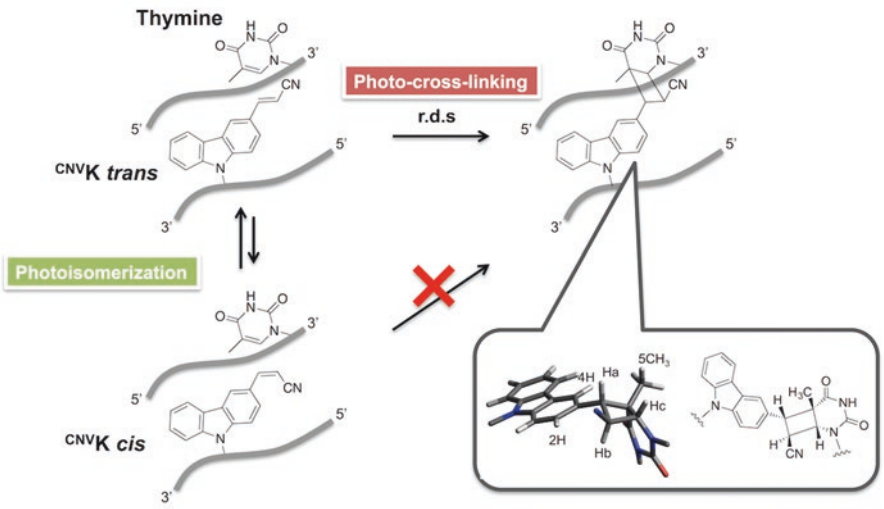


Fig. 5 Photo-cross-linking reaction of ^{CNV}K in DNA duplex

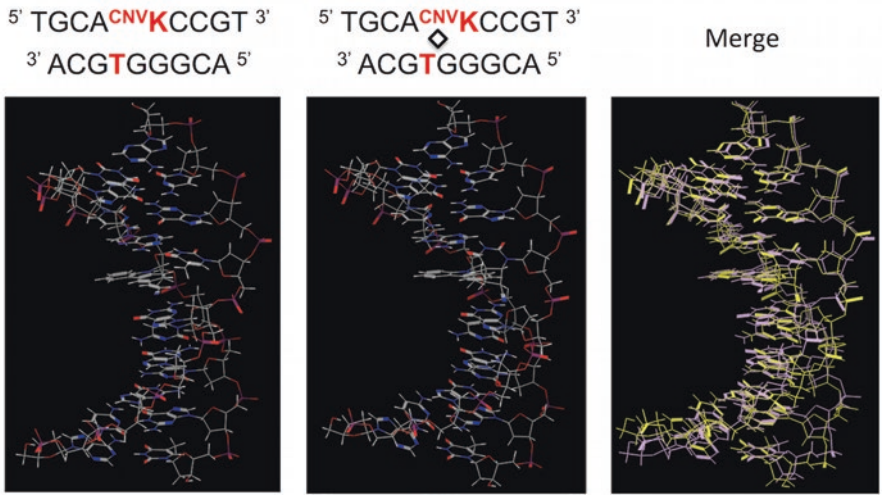


Fig. 6 Predicted energy minimized structure of the duplexes containing ^{CNV}K before and after the photo-cross-linking

4 Gene-Silencing Using ^{CNV}K Modified Antisense ODNs

Because of its ultra-fast photo-cross-linking properties, photo-cross-linkable ODNs having ^{CNV}K or ^{CNV}D might be applicable as the photoresponsive antisense ODNs for specific gene regulation through the photo-cross-linking with target mRNA. The ultra-fast photoresponse might enable us to regulate spatiotemporally the gene

expression in cells without intense photo-damage to the cells and to investigate the function of the gene of interest at various places and timings in cells. There are some reports about the photo-cross-linkable antisense ODNs using psoralen as a photo-cross-linker [12, 22]; however, the lower photoreactivity of psoralen requires long-time UV irradiation that increases photo-cytotoxicity toward living cells. In this section, the practical design, experimental procedures and how to use ^{CNV}K-modified antisense ODNs for gene silencing in the cells are described.

4.1 Design of the Photoreactive Antisense ODNs Having ^{CNV}K

Similar to the general design method of antisense ODNs, the selectivity of the duplex formation with target mRNA and the stability of the hetero-duplex is crucial to obtain the effective sequence. As in the case of ^{CNV}K-modified ODNs, hybrid stability before the photo-cross-linking is lower than that of the unmodified duplex because the 3-cyanovinylcarbazole cannot pair with any canonical bases in the RNA strand, and the position of ^{CNV}K in the antisense ODNs is important to obtain the effective sequence. ^{CNV}K should be possessed at the termini of the antisense sequence to the greatest extent possible. In our cases, highly specific photoresponsive antisense ODNs for K-ras point-mutated mRNA [27], vascular endothelial growth factor mRNA [27], and for enhanced green fluorescent protein mRNA [25] were successfully obtained. These antisense ODNs can photo-cross-link completely with their target mRNA by 1 s of 366 nm irradiation in aqueous buffer solution.

4.2 Evaluation of the Photo-Cross-Linking Reaction with mRNA

In the antisense strategy for gene silencing, the binding between antisense ODNs and target mRNA is crucial for obtaining an effective silencing effect. To evaluate quantitatively the photo-cross-linking efficiency of the ^{CNV}K-modified ODNs and target mRNA, the quantification of the reverse-transcription inhibition is effective. As the reverse-transcription reaction is stopped around the position of the photo-cross-linked hetero-duplex caused by the photoreaction of ^{CNV}K, full-length cDNA derived from the reverse-transcription is decreased with the photo-cross-linking reaction (Fig. 7). Therefore, the photo-cross-linking efficiency of the photo-reactive antisense ODNs can be evaluated quantitatively by the decrease in the amount of full-length cDNA, which can be easily quantified by a real-time PCR method with an appropriate primer set.

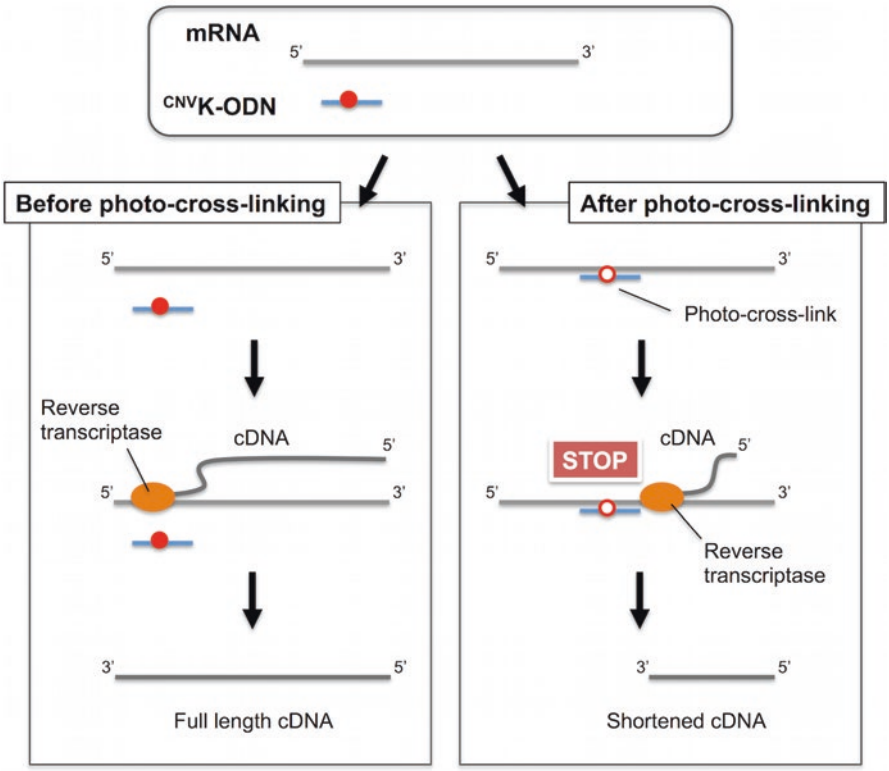


Fig. 7 Schematic drawing of the quantitative evaluation of the photo-cross-linking reaction between the ^{CNV}K-modified antisense ODNs and target mRNAs

4.3 Photo-Induced Gene Silencing in Cells

Relying on the ultra-fast photo-cross-linking manner of the 3-cyanovinylcarbazole-modified photo-cross-linkable antisense ODNs, cellular mRNA might be photo-cross-linked and the gene expression might be down regulated with a few seconds of 366 nm irradiation toward antisense-treated cells. In our study, various photo-cross-linkable antisense ODNs having ^{CNV}K were designed for silencing the GFP gene in constitutively GFP expression cells (GFP–HeLa) [25]. Since a 60% decrease of target mRNA and 40% decrease of GFP protein were observed by 10 s irradiation of 366 nm UV-LED in the case of the most effective antisense ODN (Fig. 8), the applicability of the ^{CNV}K-based photo-cross-linkable ODNs was demonstrated. In particular, multiple-irradiation increases the gene-silencing effect of the antisense ODN, and the effect was comparable to that of siRNA, suggesting that further development of this strategy might provide more sophisticated and effective gene regulation tools that can regulate genes of interest in a spatiotemporal manner.

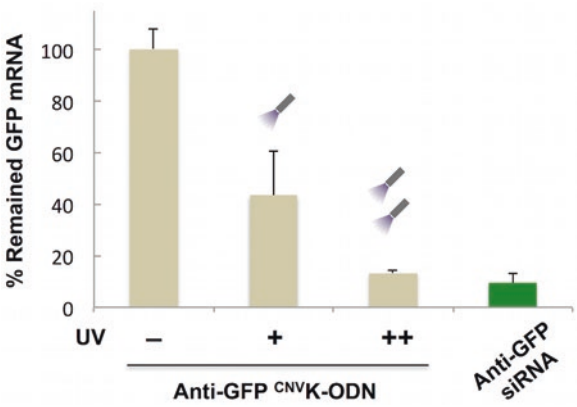


Fig. 8 Antisense effect of photo-cross-linkable antisense ODN having ^{CNV}K toward GFP expression

5 Summary

We described a practical method to synthesize and use photo-cross-linkable ODNs having ^{CNV}K and ^{CNV}D, which currently are the most highly reactive photo-cross-linkable ODNs in the world, toward gene silencing in the cells. We hope that further applications based on our photo-cross-linking technique will be developed by chemists, biologists and also medical researchers.

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