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Synthesis and properties of oligonucleotides containing fluorescent ethenodeoxyadenosine and ethenodeoxycytidine

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Abstract

Fluorescent ethenodeoxyadenosine (dA′) or/and ethenodeoxycytidine (dC′) were site-specifically inserted into four oligonucleotides through phosphoramidite chemistry on an automated DNA synthesizer, their nucleoside compositions were confirmed by enzymatic digestion analysis. The UV melting behaviors indicate that the conformation of modified duplex is not affected when incorporated into modified nucleosides. The introduction of dA′ to the oligonucleotide slightly increases the duplex stability relative to the corresponding unmodified oligonucleotide while the introduction of dC′ slightly decreases the duplex stability. Fluorescence is greatly quenched when dA′ or dC′ is inserted into an oligonucleotide and quenched further when the dA′ or dC′-modified oligonucleotide binds with its complementary DNA. The fluorescence intensity of an oligonucleotide can be increased by inserted two or more molecules of dA′.

Keywords: Fluorescent analogs; Oligonucleotide; Enzymatic digestion; UV melting curve; Fluorescence intensity; Complementary DNA

1. Introduction

Fluorescent dye is an important tool for studying nucleic acids and protein–DNA interactions. Fluorophores usually are very sensitive to changes in environment and guest molecules, and they can reflect these changes through measurable differences in fluorescence spectra. Oligonucleotide probes labeled with fluorescent dyes at their termini, which keep the affinity of oligonucleotides for their complementary segments and the sequence specificity of recognition [5], are increasingly used to studies in gene expression in situ [1], in quantitative analysis of DNA products from polymerase chain reaction assays [2], protein–DNA interactions [3], DNA sequencing and hybridization [4]. These fluorescence labeling have two disadvantage (1) the presence of one or more bulky molecule of dye, e.g. fluorescein, rhodamine and

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cyanine, would affected the hybridization; (2) the nucleosides labeled with fluorescent dye would decrease the efficiency of DNA synthesis. Therefore, we hope to explore fluorescence analysis method without using traditional fluorescent dye as label. The fluorescent analogues of nucleosides serving as two purposes of fluorescent dye and nucleobase, which have strong fluorescence and ability of hydrogen bonding with the corresponding nucleoside, become our target.

Many fluorescent nucleoside analogs and their potential utilities in studying nucleic acid dynamics [6,7] and protein–DNA interactions [8,9] have been reported. Here, we reported that the synthesis, sequence specificity for hybridization, changes in fluorescence and the affinity for DNA segments of novel modified oligonucleotides containing one or two fluorescent nucleosides at internal positions, e.g. ethenodeoxyadenosine (dA′) or/and ethenodeoxycytidine (dC′).

2. Results and discussion

Deoxyadenosine was cyclized with chloride acetaldehyde to produce ethenodeoxyadenosine (dA′), dimethoxytritylation in pyridine to give DMTr-dA′ and phosphoramidite protection in acetonitrile to afford PPT-dA′. With deoxycytidine as starting material, the ethenodeoxycytidine (dC′), DMTr-dC′ and PPT-dC′ were synthesized in the same way, respectively.

2.1. Synthesis of the modified oligonucleotides containing dA′ or dC′

The oligonucleotides containing dA′ and dC′ were synthesized by the fully automated solid phase phosphoramidite methods. Efficient coupling (98%) was achieved when a large excess of the amidite was used. The nucleoside compositions of oligonucleotides were verified by enzymatic digestion analysis and ESMS spectra (Table 2).

The nucleoside compositions of the modified oligonucleotides and the corresponding unmodified oligonucleotide are listed in Table 1.

Table 1
The compositions of the modified oligonucleotides (O-1, O-2, O-3, O-4) and the corresponding unmodified oligonuleotide (O-5)

Oligonucleotide	Sequence
O-1	3′-dGdTdGdGdTdGdTdGdA′dCdGdT-5′
O-2	3′-dGdTdGdGdTdGdTdGdAdC′dGdT-5′
O-3	3′-dGdTdGdGdTdGdTdGdA′dC′dGdT-5′
O-4	3′-dGdTdGdGdA′dGdTdGdA′dCdGdT-5′
O-5	3′-dGdTdGdGdTdGdTdGdAdCdGdT-5′

Table 2
Nucleoside molar ratio of the modified oligonucleotides found in the enzymatic digestion analysis

Oligonucleotide	dA	dC	dT	dG	dA′	dC′
O-1	–	1.0	3.7	5.6	1.0	–
O-2	1.0	–	4.2	6.2	–	1.0
O-3	–	–	4.1	6.0	1.0	0.9
O-4	–	1.0	2.9	6.1	2.1	–

2.2. Duplexes of oligonucleotides with their complementary DNA

Interactions of the modified oligonucleotides with their complementary sequences in aqueous solution were investigated spectrophotometrically. All the UV melting profiles, for the modified oligonucleotide duplexes formed with the complementary DNA, exhibited sigmoidal curves, which were similar to that for the corresponding unmodified duplexes. It indicated that the conformation of modified duplex is not affected when incorporated into modified nucleosides. The melting profiles for duplexs of O-1 and O-5 are shown as examples in Fig. 1. The melting temperature (Tm) values derived from the UV melting profiles are listed in Table 3. Inspection of the Tm values reveals that: (1) the introduction of dA′ to the oligonucleotides slightly increases the duplex stability relative to the corresponding unmodified oligomer; (2) the introduction of dC′ to the oligonucleotides slightly decreases the duplex stability relative to the corresponding unmodified oligomer.

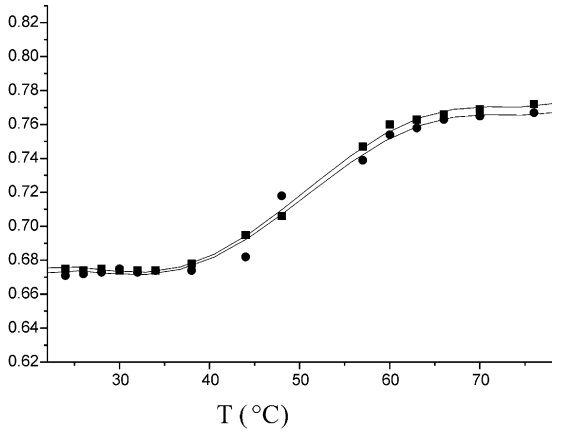

Fig. 1. UV melting curves measured at 260 nm for duplexes of oligomer O-1 (■) and unmodified oligonucleotide O-5 (●) with DNA at a common total strand concentration (1.0×10^{-5} M). The buffer (0.1 M NaCl and 0.01 M sodium phosphate) adjusted to pH 7.0.

Table 3
Spectroscopically measured Tm values for duplexes of oligonucleotides with complementary DNA[a]

Duplex	Tm (°C)
O-1 + complementary DNA	49.0
O-2 + complementary DNA	46.5
O-3 + complementary DNA	46.8
O-5 + complementary DNA	48.5

[a] Complementary DNA: 5′-dCdAdCdCdAdCdAdCdTdGdCdA-3′.

2.3. Fluorescence studies

The UV/Vis absorption and fluorescence data of etheno-deoxynucleosides and their derivatives are listed in Table 4. The experiment results showed that: (1) compounds dA′, DMTr-dA′ and PPT-dA′ have relatively high quantum yields and longer wavelength of fluorescence emission; (2) compounds dC′, DMTr-dC′ and PPT-dC′ have relatively low quantum yields and shorter wavelength of fluorescence emission.

Table 5
Fluorescence data of the modified oligonucleotides and their duplex with complementary DNA

Oligonucleotide	λ_{em} [nm]	Fluorescence ratio
O-1	414	0.273[a]
O-2	340	0.251[b]
O-3	412	0.229[a]
O-4	409	0.493[a]
O-1 + DNA	413	0.132[a]
O-2 + DNA	341	0.114[b]
O-3 + DNA	414	0.106[a]

[a] The fluorescence ratio is the relative fluorescence intensity of the oligonucleotide compared with that of compound dA′ in water ($\lambda_{ex}=300$ nm, each oligo at 1×10^{-5} M).

[b] Compared with that of compound dC′ in water ($\lambda_{ex}=300$ nm, each oligo at 1×10^{-4} M).

The fluorescence data of the modified oligonucleotides and their duplexes with complementary DNA are listed in Table 5. The experimental results showed that: (1) insertion of dA′ into an oligonucleotide greatly quenches its fluorescence intensity (72.7%). This phenomenon was very common in the labeling of fluorescent dyes, which might be due to fluorescence quenching caused by the presence of strong stacking interaction between fluorophore and the adjacent base [10]. (2) Insertion of dC′ into an oligonucleotide also greatly quenches its fluorescence intensity (74.9%); (3) insertion of dA′ into a dC′-modified

Table 4
UV/Vis absorption and fluorescence data of etheno-deoxynucleosides and their derivatives

Compound	Solvent	Absorbance λ_{max} [nm]	Fluorescence λ_{max} [nm]	Quantum yield ϕ
DA′	CH_3OH	275	408	0.510
DA′	H_2O	275	406	0.568
DMTr-dA′	CH_3OH	276	405	0.502
PPT-dA′	CH_3OH	275	406	0.278
DC′	CH_3OH	272	335	0.055
DC′	H_2O	271	342	0.053
DMTr-dC′	CH_3OH	271	336	0.057
PPT-dC′	CH_3OH	272	336	0.063

oligonucleotide (at the site beside dC′) slightly quenches its fluorescence intensity (16.1%); (4) the fluorescence intensity of the oligonucleotide containing two dA′ is almost 2-fold (181%) of that of the oligonucleotide containing one dA′; (5) binding of the dA′- or dC′-modified oligonucleotide with its complementary sequence resulted in a significant inhibition of the fluorescence (about 50%).

3. Experimental

^{1}H NMR (500 MHz) spectra were recorded on a Brucker AMX-500 NMR spectrometer. The residual proton resonance of the solvent was used as the internal standard. Chemical shifts are reported in parts per million (ppm). Infrared spectra were obtained on a Nicolet FT-IR-20SX spectrophotometer. Mass spectra were performed on Hitachi M80 (EIMS), PE Sciex API 100 (ESMS) and Brucker APEX II (TOF-MS) instruments. Elemental analyses were measured on an Italian MOD.1106 analyzer. UV/Vis absorption spectra were recorded on a Shimadu UV-265, fluorescence spectra on a Varian Cary Eclipse. HPLC analyses were performed on a Hewlett Packard 1100 system using a 4.68×250 mm Zorbax Rx-C_{18} column, a linear gradient of CH_3OH 0–70% in 60 min I water, flow rate = 1 ml/min. All reagents were provided by standard commercial suppliers.

3.1. 5,N^4-etheno-2′-deoxyadenosine (dA′)

A solution of 0.502 g (2 mmol) of 2′-deoxyadenosine in 20 ml of 2 M aqueous chloroacetaldehyde was stirred at 37 °C for 24 h and adjusted to pH 4.5–5.0 during the reaction, then the reaction mixture was evaporated to dryness in vacuo. Purification was achieved through flash-chromatography on silica-gel column (3×20 cm) in 400 ml of $CHCl_3/CH_3OH$ 4/1. The last fraction containing the product was collected, evaporated to dryness and dried under vacuum to give a white solid (0.132 g, 24%). ^{1}H NMR (500 MHz, D_2O): δ = 8.80 (s, 1H), 8.21 (s, 1H), 7.74 (s, 1H), 7.37 (s, 1H), 6.36 (t, J = 6.7 Hz, 1H), 4.58 (dt, J = 4.5, 3.5 Hz, 1H), 4.09 (dt, J = 4.5, 3.6 Hz, 1H), 3.73 (m, 2H), 2.77–2.53 (m, 2H); IR (KBr): ν = 3210 (s, OH), 1650 cm^{-1} (s, C = N); EIMS: m/z (%): 275 (4) $[M]^+$, 159 (100), 132 (15), 117 (7), 85 (17), 74 (27), 73 (30), 57 (42), 56 (21), 42 (100).

3.2. 5′-O-dimethoxytrityl-5,N^4-etheno-2′-deoxyadenosine (DMTr-dA′)

10 ml of dry pyridine was added to 0.275 g (1 mmol) of 5,N^4-etheno-2′-deoxyadenosine, and the solution was evaporated in vacuum. The evaporation was repeated three times with 10 ml of dry pyridine each time, and the residue was then suspended in 20 ml of dry pyridine. 0.406 g (1.2 mmol) of dimethoxytrityl chloride was added to this solution and the mixture was stirred at room temperature for 24 h. The solution was evaporated to give the crude product. Purification was achieved through flash-chromatography on silica-gel column (3×20 cm) in 300 ml of $CHCl_3/CH_3OH$ 4/1. The third fraction containing the product was collected, evaporated to dryness and dried under vacuum to give a yellow solid (0.30 g, 52%). ^{1}H NMR (500 MHz, CD_3COCD_3): δ = 9.13 (s, 1H), 8.33 (s, 1H), 8.04 (d, J = 1.5 Hz, 1H), 7.74 (d, J = 1.5 Hz, 1H), 6.75–7.50 (m, 13H, ArH), 6.62 (t, J = 6.5 Hz, 1H), 4.73 (dt, J = 6.1, 4.0 Hz, 1H), 4.22 (dt, J = 5.7, 4.0 Hz, 1H), 3.75 (OCH_3), 3.74 (OCH_3) 3.38 (m, 2H), 3.13–2.57 (m, 2H); IR (KBr): ν = 3210 (s, OH), 1650 cm^{-1} (s, C = N); TOF-MS: m/z: 577.3 $[M]^+$; anal. calcd. for $C_{33}H_{31}N_5O_5$: C 68.62, H 5.41, N 12.12; found C 68.48, H 5.39, N 12.14.

3.3. 3′-O-[2-cyanoethoxy(diiso-propylamino) phosphino]-5′-O-(4,4′-dimethoxytrityl)-5,N^4-etheno-2′-deoxyadenosine (PPT-dA′)

2 ml of dry acetonitrile was added to 57.7 mg (0.1 mmol) of 5′-O-dimethoxytrityl-5,N^4-etheno-2′-deoxyadenosine, and the solution was evaporated in vacuum. The evaporation was repeated three times with 2 ml of dry acetonitrile each time, and the residue was then dissolved in 5 ml of dry acetonitrile. 7.2 mg of tetrazole (0.1 mmol) and 30.1 mg (0.15 mmol) of 2-cyanoethyl bis(diisopropylaminophosphane) were added to this solution and the mixture was stirred at room temperature

under argon for 24 h. The solution was evaporated to give the crude product. Purification was achieved through flash-chromatography on silica-gel column (3×20 cm) in 400 ml of CH_2Cl_2/C_2H_5OH/Et_3N 98/2/1. The first fraction containing the product was collected, evaporated to dryness and dried under vacuum to give a yellow solid (19.4 mg, 25%). ^{1}H NMR (500 MHz, CD_3COCD_3):δ = 9.14 (s, 1H), 8.38 (s, 1H), 8.07 (s, 1H), 7.57 (s, 1H), 6.75–7.50 (m, 13H, ArH), 6.27 (m, 1H), 4.73 (m, 1H), 4.31 (m, 1H), 3.74 (m, 6H, $2OCH_3$), 3.68 (m, 2H, $POCH_2$), 3.43 (m, 2H), 2.50–3.25 (m, 6H), 1.00–1.30 [m, 12H, $4CH_3$ (iPr)]; IR (KBr):ν = 1640 cm^{-1} (s, C = N); ESMS: *m/z*: 816.4 $[M+K]^+$, 800.4 $[M+Na]^+$, 778.4 $[M+1]^+$; anal. calcd. for $C_{42}H_{48}N_7O_6P$: C 64.85, H 6.22, N 12.60; found C 64.72, H 6.24, N 12.54.

3.4. 3,N^4-etheno-2′-deoxycytidine (dC′)

0.454 g (2 mmol) of 2′-deoxycytidine was converted to a white solid in the way similar to the synthesis of dA′. (0.142 g, 28%). ^{1}H NMR (500 MHz, D_2O): δ = 8.17 (d, *J* = 8.0 Hz, 1H), 8.01 (d, *J* = 2.3 Hz, 1H), 7.72 (d, *J* = 2.3 Hz, 1H), 7.01 (d, *J* = 8.0 Hz, 1H), 6.45 (t, *J* = 6.4 Hz, 1H), 4.58 (dt, *J* = 4.5, 4.2 Hz, 1H), 4.09 (dt, *J* = 6.4, 3.5 Hz, 1H), 3.83 (m, 2H), 2.58–2.42 (m, 2H); IR (KBr):ν = 3280 (s, OH), 1740 (s, C + O), 1640 cm^{-1} (s, C = N); EIMS: *m/z* (%): 251 (1) $[M]^+$, 215 (9), 135 (73), 117 (19), 107 (34), 98 (28), 99 (21), 81 (100), 69 (21), 53 (36).

3.5. 5′-O-dimethoxytrityl-3,N^4-etheno-2′-deoxycytidine (DMTr-dC′)

0.251 g (1 mmol) of dC′ was converted to a yellow solid in the synthesis similar to DMTr-dA′ (0.30 g, 52%). ^{1}H NMR (500 MHz, CD_3COCD_3): δ = 7.77 (d, *J* = 2.3 Hz, 1H), 7.75 (d, *J* = 8.0 Hz, 1H), 7.51–6.92 (m, 14H), 6.54 (t, *J* = 6.5 Hz, 1H), 6.41 (d, *J* = 8.0 Hz, 1H), 4.58 (dt, *J* = 6.3, 4.2 Hz, 1H), 4.13 (dt, *J* = 6.3, 4.1 Hz, 1H), 3.79 (s, 6H, $2OCH_3$), 3.44 (m, 2H), 2.47 (m, 2H); IR (KBr): ν = 3320 (s, OH), 1705 (s, C = O), 1630 cm^{-1} (s, C = N); TOF-MS: *m/z*: 553.2 $[M]^+$; anal. calcd. for $C_{32}H_{31}N_3O_6$: C 69.43, H 5.64, N 7.59; found C 69.27, H 5.62, N 7.81.

3.6. 3′-O-[2-cyanoethoxy(diiso-propylamino) phosphino]-5′-O-(4,4′-dimethoxytrityl)-3,N^4-etheno-2′-deoxycytidine (PPT-dC′)

53.3 mg (0.1 mmol) of DMTr-dC′ was converted to a yellow solid in the synthesis to similar to PPT-dA′ (15.8 mg, 21%). ^{1}H NMR (500 MHz, CD_3COCD_3): δ = 7.74 (s, 1H), 7.69 (s, 1H), 6.92–7.51 (m, 14H, ArH), 6.27 (m, 1H), 6.43 (m, 1H), 4.82 (m, 1H), 4.27 (m, 1H), 3.80 (s, 6H, $2OCH_3$), 3.65 (m, 2H, $POCH_2$), 3.49 (m, 2H), 2.50–3.00 (m, 6H), 1.00–1.30 [m, 12H, $4CH_3$ (iPr)]; IR (KBr):ν = 1700 (s, C = O), 1630 cm^{-1} (s, C = N); ESMS: *m/z*: 792.4 $[M+K]^+$, 776.4 $[M+Na]^+$, 754.4 $[M+1]^+$; anal. calcd. for $C_{41}H_{48}N_5O_7P$: C 65.33, H 6.42, N 9.29; found C 65.21, H 6.39, N 9.33.

3.7. Synthesis and purification of oligonucleotides

All oligonucleotides were synthesized on a 0.2 μmol scale on a Millipore Expedite 8909 DNA synthesizer using conventional 2-cyanoethyl phosphoramidite chemistry. The modified and standard bases were dissolved in anhydrous acetonitrile (0.1 mol final concentration). The coupling time of the modified phosphoramidites were prolonged to 15 min. The coupling efficiency was the same as that of unmodified phosphoramidites (>98%). The crude oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis. Bands were detected by UV shadowing, excised and extracted into 0.3 M NaAc (pH 5.2) at 37 °C over 12 h, followed by ethanol precipitation.

3.8. UV thermal melting experiments

Purified oligonucleotides and the complementary DNA were dissolved in a phosphate buffer (pH 7.0) containing 0.1 M NaCl and 0.01 M sodium phosphate and kept at room temperature for 8 h at a common concentration (each strand: 5.0×10^{-6} M). Then the absorbances at 260 nm were measured from 20–80 °C.

3.9. Enzymatic digestion analysis of oligonucleotides

To 3.0 A_{260} unit of the oligonucleotide in 120 μL of 300 mM potassium phosphate pH 4.6 was

added 48 units of S1 nuclease. After a 6 h incubation at 37 °C, the reaction mixture was rebuffered by the addition of 120 μL of 100 mM Tris–HCl (pH 7.5), 100 mM $MgCl_2$. To this mixture was added 24 units of calf intestine alkaline phosphatase, and the mixture incubated an addition 24 h at ambient temperature. The hydrolysate was analyzed by reversed-phase HPLC using 4.68×250 mm column of Zorbax Rx-c18 and 20 mM potassium phosphate (pH 5.5) with a gradient of methanol (0–70% in 60 min).

3.10. Fluorescence measurements

The buffers for fluorescence measurements were the same as the ones used for Tm experiments. The fluorescence spectra were recorded on a Varian Cary Eclipse spectrofluorimeter.

4. Conclusions

The introduction of fluorescent dA′ to the oligonucleotides slightly increases the duplex stability relative to the corresponding unmodified oligomer while the introduction of fluorescent dC′ slightly decreases the duplex stability. Fluorescence is greatly quenched when dA′ or dC′ is inserted into an oligonucleotide and further quenched when the oligonucleotide binds with its complementary DNA. The fluorescence intensity can be increased by inserted two or more molecules of dA′. This labeling without using traditional fluorescent dyes, can be used in synthesis of DNA automatically, did not obviously affect the hybridization of oligonucleotides and can give very similar fluorescence behavior to the labeling of traditional fluorescent dyes.

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