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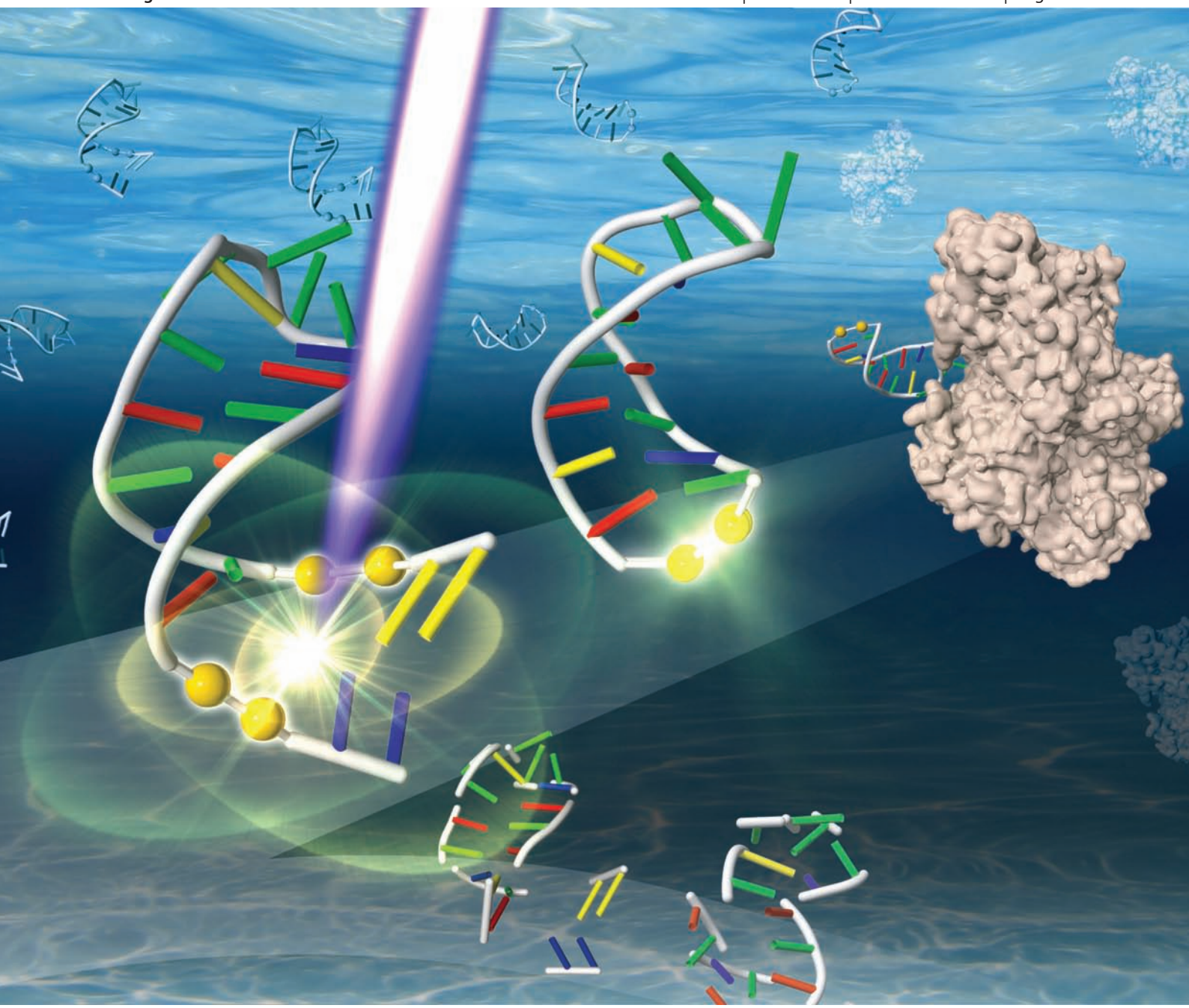

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FULL PAPER

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Radiolytic cyclization of stem-and-loop structured oligodeoxynucleotide with neighboring arrangement of α,ω-bis-disulfides†

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Upon X-ray irradiation of hypoxic aqueous solution, modified oligodeoxynucleotides (ODNs) bearing a pair of disulfides at both ends of the strand that forms a stem-and-loop structure with a neighboring arrangement of α,ω-bis-disulfides underwent efficient cyclization *via* an intramolecular exchange reaction at the disulfide moieties with a multiple turnover process. Mechanistic studies revealed that hydrogen atoms generated in the radiolysis of water are key active species initiating a chain reaction to produce cyclic ODN disulfides, in which addition of hydrogen atom results in dissociation of the original disulfide bond to generate a thiyl radical intermediate as the chain carrier for the succeeding disulfide exchange into cyclization. The properties were also assessed for the resultant cyclic ODN disulfide that has several favorable features for use in the transcriptional decoy strategy. The cyclic ODN disulfides produced by the present radiolytic method showed high thermal stability, resistance to nuclease, and high binding activity to a representative transcriptional factor of nuclear factor κB.

Introduction

DNA strands form a variety of higher order structures, such as stem-and-loop, triplex, quadruplex and cruciform besides ordinary duplexes according to specific nucleobase sequences,[1] thus they have attracted much present attention to their application as medical and diagnostic tools.[2,3] For these purposes, a great number of DNA modifications and analogs have been designed so that the higher-order structures can be rationally regulated by means of several external triggers such as metal ion binding,[4] pH or thermal change,[5] photoirradiation[6] and X-ray irradiation.[7] These artificial DNAs are potential candidates for medical application to drugs and molecular imaging probes in view of their inherent functions of molecular targeting and structural self-regulation.

The disulfide bond has been identified as a useful functional group for constructing the secondary or tertiary structure of DNA architecture.[8] Since formation and dissociation of the disulfide bond are affected significantly by the redox state, the higher-ordered DNA structures are possibly regulated by the well designed redox reaction of disulfide bond.[9] Recently, we displayed that radiolytic reduction of the disulfide bond located at a strand end of oligodeoxynucleotide (ODN) can induce ligation of ODNs *via* interstrand exchange of the disulfides in a hypoxia-selective manner.[10] This unique disulfide exchange proceeded by a chain reaction mechanism with multiple turnovers, which could be applied to the template-directed ligation[11] of two flanking ODNs.

In this study, we extended the radiolytic method of interstrand disulfide exchange for ligation of ODNs to intrastrand disulfide exchange for cyclization of ODN forming a stem-and-loop structure that causes the neighboring arrangement of disulfide bonds at both strand-ends, thereby establishing a general guide for designing radiation-associated regulation of the DNA higher-order structures. Analogous to the interstrand ligation reported previously,[10] α,ω-bis-disulfides of stem-and-loop structured ODNs in the adjacent arrangement underwent intrastrand exchange reactions to form cyclized ODNs efficiently with multiple turnovers upon hypoxic X-ray irradiation of diluted aqueous solution. Evidence was also obtained that hydrogen atoms generated from radiolysis of water are the key intermediate species involved in the cyclization. We propose herein a chain reaction mechanism, by which addition of a hydrogen atom to disulfide followed by its bond dissociation produces a thiyl radical intermediate as a chain carrier of the intramolecular cyclization of ODN with multiple turnovers. The cyclic ODN thus produced was confirmed to have several remarkable properties that would be favorable for a transcriptional decoy strategy: *e.g.*, high thermal stability, stability toward enzymatic digestion, and the ability to bind tightly to nuclear factor κB (NF-κB) as a representative transcription factor.

Results and discussion

The ODNs bearing an α,ω-bis-disulfide were synthesized by a standard automated DNA synthesis as summarized in Fig. 1. All ODNs formed stem-and-loop structures below 40 °C according to the measurement of melting temperature. We firstly conducted X-ray radiolytic reduction of 20 mer ODN 1 in Ar-purged aqueous solution containing excess 2-methyl-2-propanol as usual. The scavenger 2-methyl-2-propanol was added to suppress possible side reactions induced by oxidizing hydroxyl radicals ($OH^{\bullet}$),[12,13] which is one of the active species generated by the radiolysis of water. Fig. 2 shows a representative reaction profile of the X-ray radiolysis of ODN 1 at 0 °C under hypoxic conditions. Appearance of a large new peak in the analytical HPLC shown in Fig. 2c was attributed to the formation of cyclized ODN (ODN C1) as confirmed by overlap injection of an authentic sample in HPLC analysis: the product ODN C1 was also fractionated by repeated

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† Electronic supplementary information (ESI) available: Detailed data of photolysis of ODN 1, the reaction of ODN 1 in the presence of ODN 5 and competition assay. See DOI: 10.1039/c0ob00275e

	T_m (°C)	Sequences
ODN 1	58.2	5'-*GG*-**SS**-ACATAGTTTTCTATGT-**SS**-CC-3'
ODN 2	52.1	5'-GG-**SS**-ACATAGTTTTTTCTATGT-**SS**-CC-3'
ODN 3	44.6	5'-GG-**SS**-ACATAGTTTCTTATGT-**SS**-CC-3'
ODN 4	83.0	5'-T-**SS**-*GGAAAGTCCCC* TTTT *GGGGACTTTCC* -**SS**-A-3'
ODN 5	-	5'-GG-**SH**
ODN 6	-	^{32}P-5'-GCT *GGGGACTTTCC* ACG-3'
ODN 7	-	3'-CGA *CCCCTGAAAGG* TGC-5'

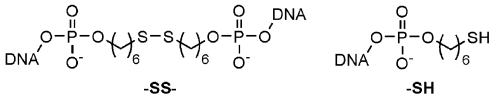

Fig. 1 Sequences, structure and melting temperature (T_m) of oligodeoxynucleotides used in this study. Stem sequences in the stem-and-loop structure are underlined. NF-κB recognition regions are shown in italic. Measurements of T_m were made in a 10 mM phosphate Na (pH 7.0) containing 100 mM NaCl.

HPLC and identified by the molecular weight measurement using ESI-TOF mass spectrometry (calcd for $[M - 3H]^{3-}$ 1749.5, found 1749.3.; calcd for $[M - 4H]^{4-}$ 1311.9, found 1311.8.). Small peaks designated as ODN X1, ODN Y1 and ODN Z1, which were formed from dinucleotide units at the strand end of ODN 1, were also observed at elution times around 24–27 min. The G values[14] were 140 nmol J^{-1} for the decomposition of ODN 1 and 100 nmol J^{-1} for the formation of ODN C1, respectively, as estimated from each time-course profile of the radiolysis (Fig. 2d).

X-Rays induce ionization and excitation of water molecules to generate electronically excited states (H_2O^*), radical cations ($H_2O^{+\bullet}$) and dry electrons (e_{dry}^-). The excited water molecules H_2O^* dissociate in a homolytic manner to hydrogen atoms ($H^\bullet$) and hydroxyl radicals ($OH^\bullet$), while radical cations $H_2O^{+\bullet}$ deprotonate to $OH^\bullet$, and dry electrons e_{dry}^- are solvated to hydrated electrons (e_{aq}^-). Eventually, e_{aq}^-, $OH^\bullet$ and $H^\bullet$ were generated as the major active species with G values of 280, 280 and 60 nmol J^{-1}, respectively, in the radiolysis of water.[13,15] The effects of these primary intermediate species on the cyclization of ODN-α,ω-bis-disulfide (Table 1) were further determined. When irradiated with X-rays in nitrous oxide (N_2O)-purged aqueous solution containing 2-methyl-2-propanol, in which hydrogen atoms $H^\bullet$ are the exclusive active species under effective scavenging of $OH^\bullet$ by 2-methyl-2-propanol and e_{aq}^- by N_2O,[12] ODN 1 was cyclized to form ODN C1 in moderate yield. In contrast, both the decomposition of ODN 1 and the formation of ODN C1 were remarkably suppressed in the radiolysis of Ar-purged aqueous solution containing 2-propanol, in which hydrated electrons e_{aq}^- are the exclusive active species under effective scavenging of $OH^\bullet$ and $H^\bullet$ by 2-propanol.[12] These results clearly indicate that the reducing species $H^\bullet$, but not e_{aq}^-, is essential for the cyclization of ODN 1 into ODN C1 *via* intrastrand exchange of α,ω-bis-disulfides, which is facilitated by the self formation of the stem-and-loop structure. A considerable number of studies have hitherto shown that one-electron reduction by e_{aq}^- leads to the S–S bond splitting and the scrambling of disulfide.[16] In this context, the present study demonstrates the importance of $H^\bullet$ in the reductive activation of the disulfide bond linked to ODN.[17] The relatively lower contribution of reducing e_{aq}^- may be attributed to the lower accessibility to its target disulfide as a consequence of the electrostatic screening effect exerted by negative charges of ODN sugar moieties.

Table 1 G-values for the decomposition of stem-and-loop structured **ODNs 1–3** and for the formation of the corresponding cyclized ODNs in the X-ray radiolysis

	ODN 1			ODN 2			ODN 3
G value (decomp.)	140[a]	140[b]	12[c]	310[d]	72[a]	110[d]	140[a]
G value (formation)	100[a]	67[b]	7.8[c]	250[d]	48[a]	63[d]	80[a]

[a] Experiments were made in an Ar-purged aqueous solution containing 50 mM 2-methyl-2-propanol at 0 °C. [b] ODN 1 was X-ray irradiated in N_2O-purged aqueous solution containing 50 mM 2-methyl-2-propanol at 0 °C. [c] ODN 1 was X-ray irradiated in aqueous solution containing 200 mM 2-propanol at 0 °C. [d] Experiments were made in an Ar-purged aqueous solution containing 50 mM 2-methyl-2-propanol at 20 °C.

In view of the evidence that the observed G values for ODN 1 decomposition (140 nmol J^{-1}) and ODN C1 formation (100 nmol J^{-1}) are larger than the G value for $H^\bullet$ generation (60 nmol J^{-1})[13] in the radiolysis of diluted aqueous solution, the cyclization of ODN 1 into ODN C1 could occur as a turnover reaction process. In the preceding study on the interstrand ligation of ODN-disulfide,[10] we assumed that the sulfide anions (ODN-S^-)

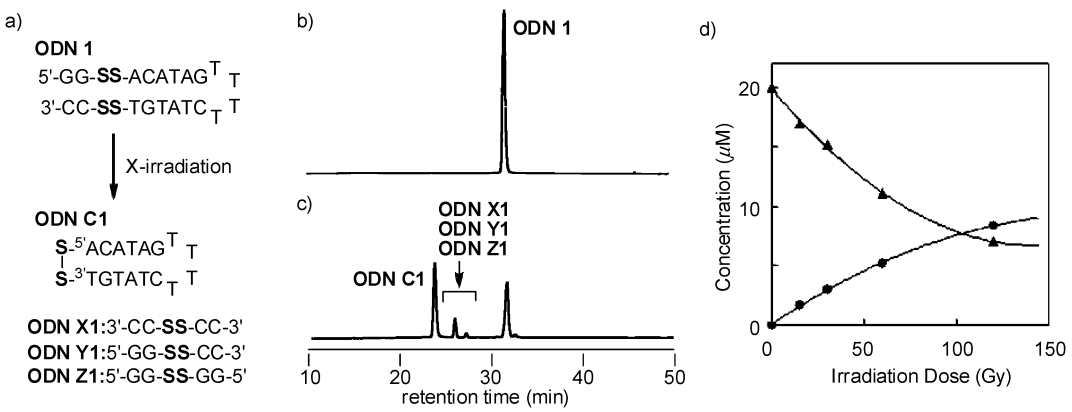

Fig. 2 (a) X-Ray irradiation of stem-and-loop structured ODN 1 to form ODN C1, ODN X1, ODN Y1 and ODN Z1. (b, c) HPLC profiles for the reaction of ODN 1 (20 μM) in the hypoxic X-ray radiolysis of aqueous solution containing 2-methyl-2-propanol (50 mM) at 0 °C: (b) before irradiation; (c) after irradiation (120 Gy). (d) The reaction profile of ODN 1 to form ODN C1 in the hypoxic radiolysis: (●) formation of ODN C1; (▲) decomposition of ODN 1.

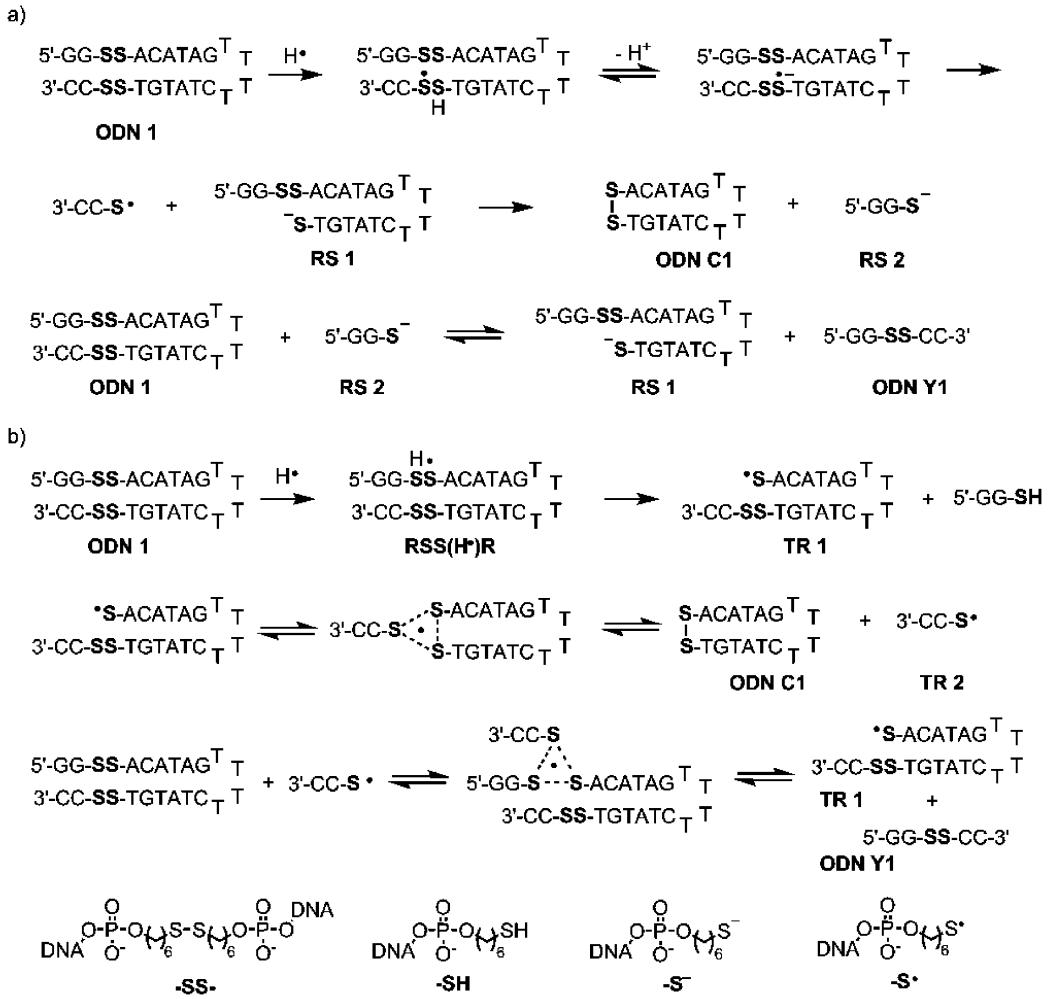

Fig. 3 Two possible reaction mechanisms for the formation of ODN C1 from ODN 1 upon X-ray irradiation: (a) sulfide anions as key intermediates. (b) Thiyl radicals as key intermediates.

formed in the radiolytic reduction of disulfide may be involved in the turnover processes as outlined in Fig. 3a. Following this mechanism, addition of H• to disulfide followed by the S–S bond splitting may be the initiation step of cyclization of ODN-α,ω-bis-disulfide to generate a sulfide anion intermediate (RS 1: ODN-S⁻). RS 1 can be a nucleophile for the disulfide bond and thereby induces an intrastrand exchange reaction of α,ω-bis-disulfides to produce ODN C1. Concurrently, an alternate sulfide anion (RS 2: 5′-GG-S⁻) is generated to be a nucleophile in a similar manner as RS 1. Thus, multiple turnovers of the nucleophile sulfide anions RS 1 and RS 2 may account for the efficient cyclization of ODN 1. To confirm the validity of this radiolytic cyclization mechanism, we also conducted a control experiment using ODN 5 (5′-GG-SH) possessing a thiol group at the 3′-end that may deprotonate to generate a presumable intermediate RS 2 (5′-GG-S⁻) in basic aqueous solution. When ODN 1 was treated with ODN 5 in neutral aqueous solution as in the case of usual X-ray radiolysis, the formation of ODN C1 was negligible and only a small amount of dimerized ODN 5, an oxidized form of thiol ODN Z1 (5′-GG-S–S-GG-5′), was yielded (Fig. S1†). A similar result was also obtained even from basic aqueous solution at pH 8.7 of ODN 1 and ODN 5, where the prompt deprotonation of thiol ODN 5 into sulfide anion RS 2 could occur. These control experiments suggest that the sulfide anion RS 2 is unlikely to be a chain carrier involved in the efficient cyclization of ODN 1 with multiple turnovers.

Several reports have previously shown that not only sulfide anions RS⁻ but also thiyl radicals (RS•) induce the intermolecular exchange reaction of disulfide compounds to form new disulfide bonds. Gupta and Knight reported that the liquid phase photolysis of an alkyl disulfide leads to disulfide bond splitting to form thiyl radicals, which participate in the efficient exchange reaction with the parent disulfide molecules.[18] Asmus and Bonifacic identified a three-electron bonded intermediate formed in the thiyl radical-induced scrambling of disulfides by means of pulse radiolysis.[19] To confirm the possible participation of the thiyl radicals as a chain carrier in the present intrastrand cyclization of ODN-α,ω-bis-disulfide, we also conducted the photolysis of ODN 1 that induces homolytic splitting of the disulfide bond into thiyl radicals. Upon photoirradiation of ODN 1 for 9 h in Ar-purged phosphate buffer solution at pH 7.0, cyclization of ODN 1 proceeded to afford ODN C1 (Fig. S2†). This is a strong indication that reactive thiyl radical intermediates are responsible for the efficient formation of ODN C1. Thus, a more likely mechanism by which the stem-and-loop structured ODN 1 undergoes radiolytic

cyclization to produce ODN C1 is outlined in Fig. 3b. The initial step involves addition of H• to disulfide to form an adduct radical intermediate (RSS(H•)R) that decomposes into thiol (5′-GG-SH) and dinucleotide thiyl radical (TR 1: ODN-S•). TR 1, derived from S–S bond splitting of disulfide at the one strand end of ODN 1, possibly forms a three-electron bonded intermediate with another neighboring disulfide bond located at the opposite strand end, through which intrastrand exchange reaction of disulfides may proceed to produce ODN C1 along with regeneration of dinucleotide thiyl radical TR 2. The turnover of TR 2 as a chain carrier accounts for the efficient cyclization of ODN-α,ω-bis-disulfide.

In the separate experiments, we attempted the X-ray radiolytic reduction of 22 mer ODN 2 possessing a 6 mer TTTTTT loop region. Similar to the 20 mer ODN 1 possessing a 4 mer TTTT loop region, the cyclization of ODN 2 proceeded upon hypoxic X-ray irradiation at 0 °C, while becoming less efficient apparently with increased ODN chain length (Table 1). By reference to other evidence that 20 mer ODN 3 possessing a 6 mer TTTTCT loop region cyclized in good yield, it appears that the lower cyclization efficiency of ODN 2 is not attributable to the effect of the expanded loop region, but is more likely caused by the increased number of thymine bases undergoing reduction by H• in competition with disulfides: among 4 the nucleobases, thymine is known to be readily reduced by H• ($k = 5.7 \times 10^8$ M^{-1} s^{-1}).[13] Incidentally, similar X-ray radiolytic reduction of ODNs 1 and 2 at 20 °C revealed that cyclization efficiency can be improved by elevating the reaction temperature.

Recently, end-capped DNAs have been widely used in the field of gene therapy because of its high stability under biological conditions.[2] Thus, further attempts were made to apply the radiation chemically cyclized ODN to a decoy strategy of directly targeting transcription factors. We prepared a 28 mer stem-and-loop structured ODN 4 bearing α,ω-bis-disulfide bonds to characterize the radiation chemical reactivity, stability, and binding properties against NF-κB. Similar to the experimental results described above, hypoxic X-ray irradiation efficiently induced the cyclization of ODN 4 (Fig. 4a–c). The stability of ODNs was determined by monitoring melting temperature (T_m) in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl (Fig. 5a). Compared to the parent ODN 4 (T_m = 83 °C), the cyclized ODN C4 (T_m > 90 °C) showed a dramatic increase in thermal stability,[20] indicating that the cyclic structure with disulfide linkage of ODN C4 may be effective for restriction of the conformational flexibility. We also investigated the resistance of ODN C4 to enzymatic digestion. As shown in Fig. 4b–d, a mixture of ODN 4 and ODN C4 prepared by hypoxic X-ray irradiation was treated with snake venom phosphodiesterase that degrades DNA from the 3′-end. Whereas prompt digestion was observed for ODN 4 under these conditions, ODN C4 was almost totally resistant to the enzymatic digestion. This indicates that the cyclized ODN has a remarkably improved stability under biological conditions. In light of the unique properties as mentioned above, an *in vitro* competition assay was further conducted to identify whether ODN C4 can interact with NF-κB as a decoy. Upon hypoxic X-ray irradiation with total doses up to 30 Gy at ambient temperature, ODN 4 was almost quantitatively converted to ODN C4, and the irradiated sample was added to constant amounts (5 nM) of ^{32}P-labeled double stranded ODN 6/ODN 7 as the NF-κB probe. The resulting mixture was incubated with NF-κB to identify the binding ability of decoy ODN with NF-κB using an electrophoretic mobility shift assay. As shown in Fig. 5b, addition of the irradiated sample, in which ODN 4 was almost quantitatively converted to ODN C4, decreased the intensity of the retarded band characteristic of the complex between ^{32}P-labeled ODN 6/ODN 7 duplex and NF-κB. The half maximal inhibitory concentration (IC_{50}) for purified ODN C4 was estimated as 54 nM (Fig. S3†). Since addition of a control ODN C1 without specified base sequence for binding to NF-κB could not compete with the ODN 6/ODN 7 duplex as the NF-κB probe, we concluded that cyclic ODN C4 can bind efficiently to NF-κB in a sequence-dependent manner.

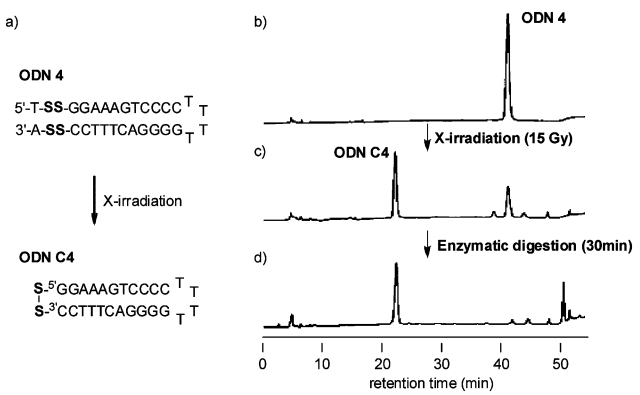

Fig. 4 (a) X-Ray irradiation of stem-and-loop structured ODN 4 to form ODN C4. (b,c,d) HPLC profiles for radiolysis and enzymatic digestion: (b) before the X-ray radiolysis of ODN 4; (c) hypoxic X-ray irradiation (15 Gy) of ODN 4 to form ODN C4 at ambient temperature; (d) enzymatic digestion with snake venom phosphodiesterase at 37 °C for 30 min of the sample obtained from radiolysis of ODN 4.

Conclusion

In summary, we characterized the radiolytic reduction of α,ω-bis-disulfide bonds incorporated into stem-and-loop structured ODNs. Hypoxic X-ray irradiation of ODNs in aqueous solution efficiently induced cyclization *via* intrastrand exchange of the disulfide bonds. According to the X-ray radiolytic reaction profiles under various conditions, the following reductive cyclization mechanism was proposed: addition of H•, which is one of the reactive species generated by the radiolysis of water, to the disulfide bond occurs in the initial step to cause S–S bond splitting and thereby generate a thiyl radical that is a key intermediate as a chain carrier for cyclization of ODN-α,ω-bis-disulfide with multiple turnovers.

In addition, the cyclic ODN compound obtained by the X-ray radiolytic reduction of ODN-α,ω-bis-disulfide showed high stability against enzymatic digestion and high binding properties against a transcription factor of NF-κB in a sequence dependent manner. The present simple method of cyclic ODN synthesis would be effective for preparation of decoy molecules, which can control gene expression by inhibition of specific transcription-regulation proteins.

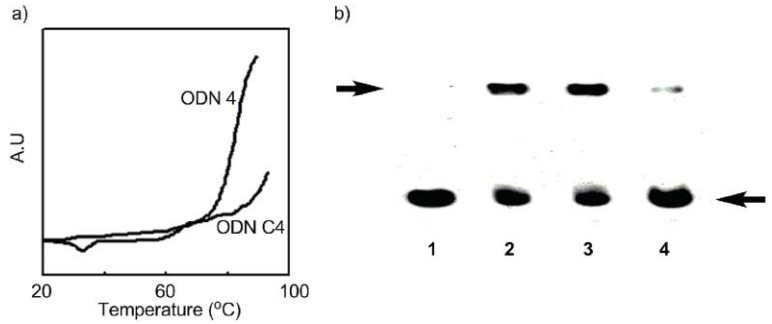

Fig. 5 (a) Melting profile of ODN 4 and ODN C4. (b) Competition assay for identification of binding properties of cyclized ODN with NF-κB. NF-κB-double stranded ODN complexes were formed between ^{32}P-5′-end labeled duplex (ODN 6/ODN 7) and the NF-κB protein (1.0 gsu) in the presence of unlabeled ODN (cyclized ODN) as the competitors: arrows indicate (left) the NF-κB complex and (right) free labeled duplex; lane 1, labeled ODN 6/ODN 7 only; lane 2, labeled ODN 6/ODN 7 + NF-κB; lane 3, labeled ODN 6/ODN 7 + NF-κB + control ODN without recognition site of NF-κB (ODN C1); lane 4, labeled ODN 6/ODN 7 + NF-κB + X-ray irradiated ODN 4 (ODN C4).

Experimental section

General methods

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry of oligonucleotides was performed on JEOL LMS-ELITE MALDI-TOF MASS spectrometer with 2′,3′,4′-trihydroxyacetophenone as matrix. ESI-TOF mass spectra were obtained on BRUKER DALTONICS micrOTOF focus-KE. The oligonucleotides were purchased from Invitrogen. The reagents for the DNA synthesizer such as A, T, G, C and thiol-modifier C6 S–S were purchased from Glen Research. Snake venom phosphodiesterase I was purchased from ICN. High-performance liquid chromatography (HPLC) was performed with a HITACHI L-2400 HPLC system. Sample solutions were injected on a reversed phase column (Inertsil ODS-3, GL Sciences Inc., ϕ 4.6 mm × 150 mm or 10 mm × 150 mm). The solvent mixture of 0.1 M triethylamine acetate (TEAA) at pH 7.0 and 100% acetonitrile was delivered as mobile phase at a flow rate of 0.6 mL or 3.0 mL min^{-1} at 25 °C. The column eluents were monitored by the UV absorbance at 254 nm or 260 nm. Rigaku RADIOFLEX-350 was used for X-ray radiolysis. Gel electrophoresis was carried out on an ATTO AE-6155 apparatus. All aqueous solutions were prepared using purified water (YAMATO, WR600A).

Synthesis of oligodeoxynucleotides. ODNs bearing a disulfide bond were synthesized by the conventional phosphoramidite method using an Applied Biosystems 392 DNA/RNA synthesizer. Synthesized ODNs were purified by reversed phase HPLC on a Inertsil ODS-3 column (10 × 250 mm, elution with a solvent mixture of 0.1 M triethylammonium acetate (TEAA) at pH 7.0, linear gradient over 40 min from 0% to 30% acetonitrile at a flow rate 3.0 mL min^{-1}). Mass spectra of ODNs purified by HPLC were determined with ESI-TOF mass spectrometry (for ODN 4) or MALDI-TOF mass spectrometry (for ODN 1, 2, 3 and 5) with 2′,3′,4′-trihydroxyacetophenone as matrix, using T_8 ([M – H]$^-$ 2370.61) and T_{17} ([M – H]$^-$ 5108.37) as an internal standard; ODN 1, *m/z* 6754.6 (calcd for [M – H]$^-$ 6753.8); ODN 2, *m/z* 7363.4 (calcd for [M – H]$^-$ 7362.2); ODN 3, *m/z* 6754.3 (calcd for [M – H]$^-$ 6753.8); ODN 4, *m/z* 1844.3 (calcd for [M – 5H]$^{5-}$ 1844.5), 1536.8 (calcd for [M – 6H]$^{6-}$ 1536.9), 1317.1 (calcd for [M – 7H]$^{7-}$ 1317.2), 1152.4 (calcd for [M – 8H]$^{8-}$ 1152.4), 1024.2 (calcd for [M – 9H]$^{9-}$ 1024.3); ODN 5, *m/z* 791.8 (calcd for [M – H]$^-$ 791.6).

Radiolytic reduction. To establish hypoxia, aqueous solutions of ODNs (20 μM) in 10 mM phosphate Na buffer (pH 7.0) containing 100 mM NaCl and 50 mM 2-methyl-2-propanol were purged with Ar for 10 min, and then irradiated in a sealed glass ampoule at ambient temperature with an X-ray source (6.0 Gy min^{-1}). After the X-ray irradiation, the solution was immediately subjected to HPLC. The formation of cyclized ODNs were identified by overlap injection of authentic samples in HPLC analysis and ESI-TOF mass spectrometry.

The control experiments, such as the reaction in the presence of 2-propanol (200 mM) or the reaction in N_2O-purged aqueous solution, were conducted in a similar manner.

Reaction of ODN 1 in the presence of ODN 5. To an aqueous solution of ODN 1 (20 μM) in 10 mM phosphate Na buffer (pH 7.0 or pH 8.7) containing 100 mM NaCl and 50 mM 2-methyl-2-propanol, ODN 5 (5 μM) was added and the resulting mixture was incubated at 20 °C. After the incubation, the solution was immediately subjected to HPLC.

Photoreaction of ODN 1. Ar-purged aqueous solution of ODN 1 (20 μM) in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl was exposed to 254 nm UV light with Handy UV Lamp SLUV-4 for 9 h at ambient temperature. The solution was subjected to HPLC immediately after the photoirradiation.

Melting temperature (T_m) measurement. T_ms of the duplexes (1 μM, duplex concentration) were taken in a 10 mM phosphate Na (pH 7.0) containing 100 mM NaCl. Absorbance *vs.* temperature profiles were measured at 260 nm using a JASCO V-530 UV/VIS spectrophotometer connected with ETC-505T temperature controller. The absorbance of the samples was monitored at 260 nm from 4 °C to 90 °C with a heating rate of 1 °C min^{-1}. From these profiles, first derivatives were calculated to determine T_m values.

Enzymatic digestion of the mixture of ODN 4 and ODN C4. To obtain the mixture of ODN 4 and ODN C4, the aqueous solution of ODN 4 (20 μM) in the presence of 2-methyl-2-propanol (50 mM) was X-ray irradiated under hypoxic conditions at ambient

temperature. The mixture of ODN 4 and ODN C4 was treated with snake venom phosphodiesterase I (0.6 mU/μL), at 37 °C for 30 min. The solution was subjected to HPLC immediately after the incubation.

Preparation of 5′-^{32}P-end labeled ODN 6. ODN 6 (60 pmol strand concentration) was labeled by phosphorylation with 3 μL of [γ-^{32}P]ATP and 5 μL of T4 polynucleotide kinase using standard procedures.[21,22] The 5′-end-labeled ODNs were purified by means of BioRad MicroBioSpin P6 column.

Competition assay. ^{32}P-5′-end labeled ODN 6/ODN 7 duplex (<5 nM strand concentration), NF-κB p50 homodimer (Promega Co., 1.0 gel shift unit) and decoy ODNs in HEPES buffer (10 mM, pH 7.0) containing $MgCl_2$ (10 mM), LiCl (50 mM), NaCl (100 mM), spermidine (1 mM), BSA (0.2 mg ml^{-1}), poly(dI:dC) (0.1 μg), IGEPAL-CA630 (0.05%), and glycerol (10%) were incubated at room temperature for 30 min. The mixture was loaded on 8% native polyacrylamide gel, electrophoresed at 600 V (4 °C) for 60 min, and then transferred to a cassette to be stored at −80 °C with Fuji X-ray film (RX-U).

The half maximal inhibitory concentration (IC_{50}) was estimated from the band intensity of ^{32}P-labeled duplex and NF-κB complex.

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