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Oxidation of Oligonucleotide-bound Ce^{III}/Multiphosphonate Complex for Site-Selective DNA Scission

Tuomas Lönnberg, Yuichiro Aiba, Yuya Hamano, Yoshitaka Miyajima, Jun Sumaoka, and Makoto Komiyama *[a]

Abstract: Oligodeoxyribonucleotide conjugates of ethylenediamine-*N*,*N*,*N*′,*N*′-tetrakis(methylenephosphonic acid) (EDTP) have been used to place a Ce^{III}/EDTP complex in close proximity of predetermined phosphodiester linkages of a complementary target oligonucleotide. In the presence of atmospheric oxygen, the Ce^{III} is oxidized into Ce^{IV} which, in turn, efficiently cleaves the target phosphodiester linkage. Two equivalents of the Ce^{III} salt to the substrate DNA and the cutting oligonucleotide-EDTP conjugate is sufficient for full catalytic activity with hardly any cleavage observed at the other single-stranded regions, suggesting that the catalytic Ce species is strictly localized next to the target phosphodiester linkage. No rate retardation is observed upon introduction of scavengers for hydroxyl radicals (DMSO or MeOH) or singlet oxygen (NaN_3) to the system, indicating that the reaction proceeds via a hydrolytic pathway. Any significant contribution by an oxidative pathway is further ruled out by the observation that nucleosides remain intact after incubation with Ce^{IV}:EDTP for extended periods.

Keywords: DNA • cleavage • hydrolysis • Ce^{III} • Ce^{IV}

Introduction

Preparation of man-made cutters for site-selective scission of DNA has been one of the most attractive and challenging themes of bio-organic chemistry, mainly because of their potential applications in advanced molecular biology, therapy, and many other fields.[1,2] Many elegant systems utilizing oxidative cleavage of the target DNA have already been developed and used for various purposes (e.g., structural probes of DNA/protein or DNA/drug interactions, as well as inhibition of target gene expression). Artificial hydrolytic cutters, which cut DNA at a desired site through hydrolysis of the target phosphodiester linkage, on the other hand, have been limited in number despite great demand for cleaving agents that are directly compatible with enzymatic DNA manipulation used in current molecular biology and biotechnology.

Among the metal catalysts reported to hydrolyze the immensely stable phosphodiester linkages in DNA, Ce^{IV} ion is characterized by its high scission activity, being able to hydrolyze DNA under physiological conditions.[3] In previous papers,[4] single-stranded DNA substrate was selectively cut at the target site by mixing Ce^{IV} salts (e.g., $Ce(NH_4)_2(NO_3)_6$) with oligonucleotide-ligand conjugates (or peptide nucleic acid-ligand conjugates), thus placing the Ce^{IV} complexes at a predetermined site (Figure 1b). In these artificial cutters, however, large excess of the Ce^{IV} salt to the conjugate (e.g., 100-1000 fold) is usually needed for efficient cleavage, owing to competitive formation of the hydroxide gel of Ce^{IV} around pH 7.[5,6] The large excess of Ce^{IV}, in turn, gives rise to off-target scission at other single-stranded regions of the target DNA. Clearly, further improvements in site-selectivity and scission-efficiency are required for more versatile and practical applications of these tools.

This paper describes a novel strategy for the preparation of well-characterized Ce^{IV}-based site-selective cutters for single-stranded DNA scission. As depicted in Figure 1a, an easily tractable Ce^{III} salt, which is markedly less prone to gel formation, is used as a precursor of the catalytically active Ce^{IV} species. As a ligand, ethylenediamine-*N*,*N*,*N*',*N*'-tetrakis(methylenephosphonic acid) (EDTP) is employed, since it can strongly bind both Ce^{III} and Ce^{IV} to prevent Ce^{IV} ions from aggregating and to recruit them to the cleavage site.[7] The Ce^{IV}/EDTP complex for site-selective DNA hydrolysis is prepared by oxidizing the Ce^{III}/EDTP complex which is bound to an oligodeoxyribonucleotide (ODN). The resultant Ce^{IV}/EDTP-ODN complex is free from undesired formation of Ce^{IV} hydroxide gel observed in previously reported Ce^{IV} salt-derived DNA cutters, and shows clear-cut site-selective scission.

[a] Dr. T. Lönnberg, Mr. Y. Aiba, Mr. Y. Hamano, Dr. Y. Miyajima, Dr. J. Sumaoka, Prof. M. Komiyama
Research Center for Advanced Science and Technology
The University of Tokyo
4-6-1 Komaba, Meguro-ku, Tokyo, 153-8904 (Japan)
Fax: (+81) 03-5452-5209
E-mail: komiyama@mkomi.rcast.u-tokyo.ac.jp

[b] Title(s), Initial(s), Surname(s) of Author(s)
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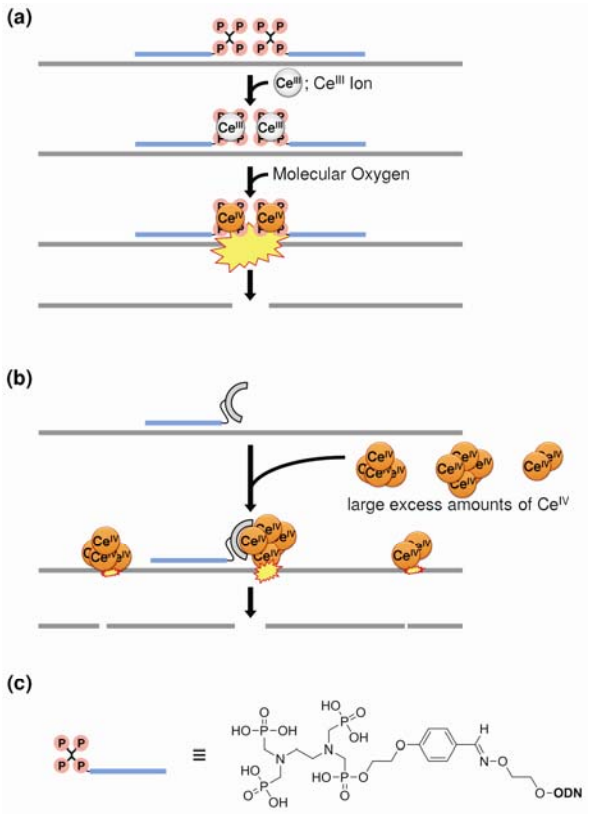

Figure 1. (a) The strategy developed in this paper for the *in situ* preparation of site-selective DNA cutters from Ce^{III} salt. A Ce^{III} salt is first added to EDTP-ODN conjugate, and the resultant Ce^{III} complex is then oxidized to the corresponding Ce^{IV} complex. Minimized unbound catalytic species gives rise to clear-cut site-selective scission. Selective scission is achievable even with the use of only one EDTP-ODN conjugate (see text for details). (b) Previously employed methods. A Ce^{IV} salt is directly added to ligand-ODN conjugate. In order to form the active Ce^{IV} complex in the presence of competitive formation of Ce^{IV} hydroxide gel, a large excess of Ce^{IV} (e.g., 100-1000 fold) is necessary, leading to off-target scission. (c) Structure of EDTP-ODN conjugate used in (a).

Results and Discussion

According to the previously described procedure,[8] an EDTP ligand was attached to either the 5′- or the 3′-end of an ODN (Figure 1c). By using two of these EDTP-ODN conjugates, a five-base gap for site-selective scission was formed in the middle of a single-stranded DNA substrate (see Figure 1a).[5] The DNA hydrolysis was started by adding $Ce(NO_3)_3$ to the mixture and carried out at 50 °C and pH 7.0 for 20 h under air. Rapid oxidation of the Ce^{III} in its EDTP complex to Ce^{IV} under these conditions was confirmed by the decrease in the intensity of luminescence from the Ce^{III} in the 350-450 nm region (see Figure 2; Ce^{IV} is emission-silent). About 90 % of the Ce^{III} was converted to Ce^{IV} in 1.5 h, and ultimately the oxidation was almost complete. No turbidity was observed in the solutions throughout the oxidation.

Figure 3 shows the PAGE patterns for the hydrolysis of **DNA$^{(S1)}$** (85- mer) using various combinations of ODN additives (20-mers in Figure 3a). In lane 5, 1:1 combination of **ODN$_1$-EDTP** and **EDTP-ODN$_2$** was used to place a Ce^{IV}/EDTP complex on both sides of the gap. Notable scission selectively occurred at the 5-base gap-site (T41-G45), yielding five bands corresponding to the scission of each phosphodiester linkage. The scission in the middle of the gap was especially strong (the total conversion of the scission was approximately 13%). The DNA scission rate steeply increased with increasing $[Ce(NO_3)_3]$, and attained a plateau when the mole ratio of $Ce(NO_3)_3$ to each of the EDTP-ODN conjugates was around 2-4 (Figure 3c).[9] Increasing $[Ce(NO_3)_3]$ beyond this saturation level resulted in off-target scission, although cleavage of the target site was still favoured (lane 9, Figure 3c). As expected, no scission occurred when all the procedures were carried out under nitrogen, because the oxidation of the Ce^{III} to Ce^{IV} was impossible (data not presented). When $CeCl_3$ was used in place of $Ce(NO_3)_3$, essentially the same results were obtained. With the use of $Ce(NH_4)_2(NO_3)_6$ in place of $Ce(NO_3)_3$, however, no site-selective scission was observed. Apparently, gel formation of the Ce^{IV} ions from the Ce^{IV} salt outcompeted the complex formation with the EDTP. The Ce^{III} salt-derived DNA cutters are thus far more clear-cut.

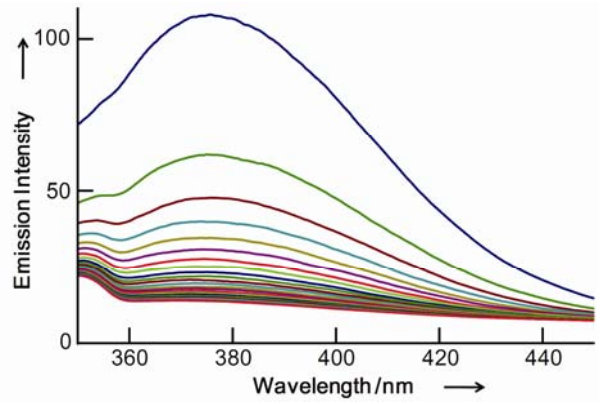

Figure 2. Change of the emission spectrum of a mixture of $Ce(NO_3)_3$ and EDTP upon incubation under air at pH 7.0 and 50 °C; $[Ce(NO_3)_3]$ = 8 μM, [EDTP] = 2 μM in 5 mM HEPES buffer ([NaCl] = 100 mM). Immediately after the preparation of the sample (the blue line), the spectra were taken at 10 min intervals (from the top to the bottom).

In lanes 6 and 7 of Figure 3b, only one Ce^{IV}/EDTP complex was placed on either the 5′- or the 3′-side of the gap (the **ODN$_1$-EDTP/H-ODN$_2$** and the **ODN$_1$-H/EDTP-ODN$_2$** combinations, respectively). The site-selective scission was still successful, although the scission efficiency was lower than that in lane 5. It is noteworthy that, in lanes 5-7 in Figure 3b, neither of the overhang regions of **DNA$^{(S1)}$** (from T1-T20 and T66-C85) was cleaved although these portions were also single-stranded. Apparently, the present site-selective scission is primarily ascribed to "proximity effect": the catalytically active Ce^{IV}/EDTP complexes are strictly localized at the target site, and thus off-target scission is avoided.

In contrast with these successful site-selective DNA scissions, virtually no scission occurred under the same conditions when no EDTP group was bound to the ODN (**ODN$_1$-H/H-ODN$_2$** combination in lane 4 of Figure 3b). Even when one monophosphate group was attached to either or both of these two ODN additives (**ODN$_1$-P** and/or **P-ODN$_2$**) to recruit the catalytic Ce species to the cleavage site, no scission was observed (lanes 8-10). Superiority of EDTP as the ligand for the site-selective DNA scission by Ce^{III}-derived Ce^{IV} is, hence, evident. In the absence of EDTP, the Ce^{IV} species, formed by *in situ* oxidation of Ce^{III}, rapidly aggregates and thus its local concentration at the desired scission site is only marginal.

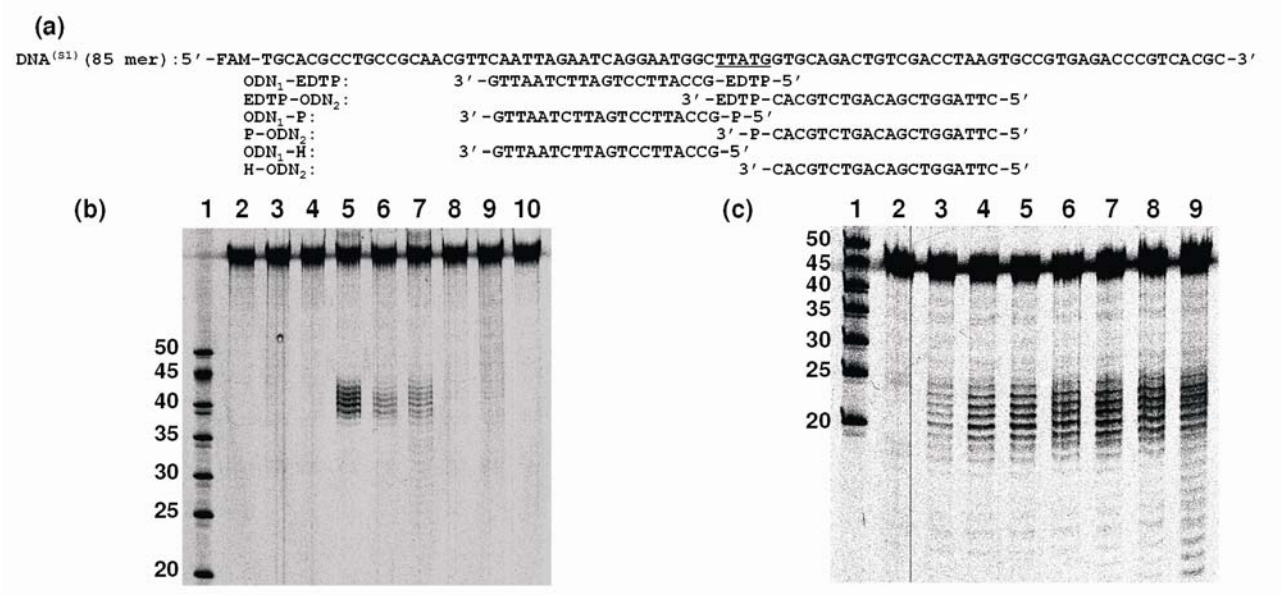

Figure 3. Site-selective DNA scission by using Ce^{III}-derived DNA cutters. (a) The substrate and the ODN additives used in this study. The gap-site is underlined. (b) Denaturing 20%-PAGE patterns for site-selective scission of **DNA**$^{(S1)}$ using various combinations of ODNs. Lane 1, Marker; Lane 2, DNA only; Lane 3, Ce^{III} only (without ODN additives); Lane 4, **ODN$_1$-H/H-ODN$_2$**; Lane 5, **ODN$_1$-EDTP/EDTP-ODN$_2$**; Lane 6, **ODN$_1$-EDTP/H-ODN$_2$**; Lane 7, **ODN$_1$-H/EDTP-ODN$_2$**; Lane 8, **ODN$_1$-P/P-ODN$_2$**; Lane 9, **ODN$_1$-P/H-ODN$_2$**; Lane 10, **ODN$_1$-H/P-ODN$_2$**. Reaction conditions: [**DNA**$^{(S1)}$] = 1 μM, [each of ODN additives] = 1 μM, [$Ce(NO_3)_3$]= 4 μM, [HEPES (pH 7.0)] = 5 mM, and [NaCl] = 100 mM at 50 °C under air for 20 h. (c) Dependence of the rate of site-selective DNA scission on the concentration of $Ce(NO_3)_3$. Reactions were carried out under the conditions mentioned above except for the concentration of $Ce(NO_3)_3$: [$Ce(NO_3)_3$]/ [EDTP-ODN] = 0 (lane 2), 1 (lane 3), 1.5 (lane 4), 2 (lane 5), 3 (lane 6), 4 (lane 7), 5 (lane 8), and 10 (lane 9), In order to simplify the system, **DNA**$^{(S2)}$ (5'-FAM-CAATT-AGAATCAGGAATGGCTTATGGTGCAGACTGTCGACCTAAG) was used as substrate.

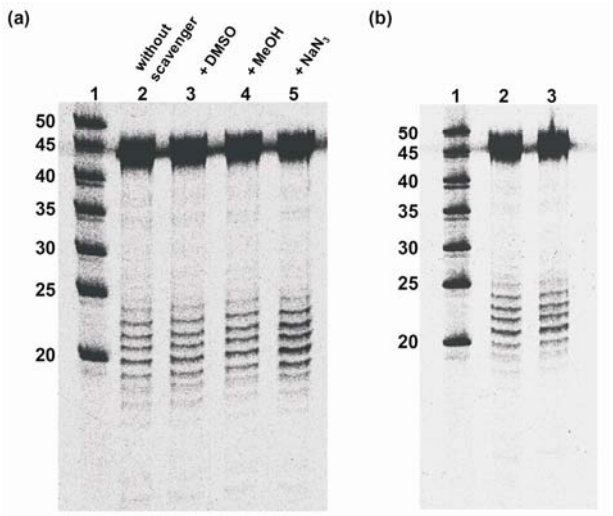

Figure 4. (a) Absence of rate-retarding effect of radical or singlet oxygen scavengers on the site-selective scission by Ce^{III}-derived DNA cutter. Lane 1, Marker; Lane 2, in the absence of scavengers; Lane 3, with 100 mM DMSO; Lane 4, with 2.5 M MeOH; Lane 5, with 10 mM NaN_3. Reaction conditions: [**DNA**$^{(S2)}$] = 1 μM, [each of ODN additives] = 1 μM, [$Ce(NO_3)_3$] = 8 μM, [HEPES (pH 7.0)] = 5 mM and [NaCl] = 100 mM at 50 °C for 20 h under air. (b) Effect of oxidation of Ce^{III} to Ce^{IV} prior to site-selective DNA scission. Lane 1, Marker; Lane 2, in situ oxidation of Ce^{III} to Ce^{IV} ($Ce(NO_3)_3$ was directly added to the reaction mixture of DNA scission under air; positive control); Lane 3, pre-oxidation of Ce^{III} to Ce^{IV} ($Ce(NO_3)_3$ was first incubated with EDTP-ODNs for 3 h under air for the oxidation, and then the **DNA**$^{(S2)}$ was added).

To assess the possibility of an oxidative cleavage mechanism, the reactions were also carried out in the presence of various scavengers for oxidation species (Figure 4a). With 100 mM DMSO (a scavenger of hydroxyl radicals), 2.5 M MeOH (also a scavenger of hydroxyl radicals), or 10 mM NaN_3 (a scavenger of singlet oxygen),[10] no measurable retardation was observed. It was, hence, confirmed that neither hydroxyl radical nor singlet oxygen plays an important role in the present DNA scission. In Figure 4b, $Ce(NO_3)_3$ was first incubated with EDTP-ODN under air at pH 7.0 and 50 °C for 3 h, during which time almost all (99%) of the Ce^{III} was oxidized to Ce^{IV}. Then, the DNA substrate was added to the mixture. Both the scission efficiency and the selectivity (lane 3) were essentially the same as without the pre-oxidation procedure (lane 2). Thus the possibility that free radicals, formed *in situ* by Fenton-like chemistry, cleave DNA *via* an oxidative pathway was further ruled out (note that Ce^{III} is required to produce a hydroxyl radical in Fenton-like chemistry).

The argument against an oxidative cleavage mechanism was further borne out by experiments involving incubation of nucleosides with either Ce^{III}-derived Ce^{IV}/EDTP (Figure 5a and b) or Fe^{II}/EDTA (Figure 5c). Relatively high catalyst and substrate concentrations were used to compensate for the fact that the effective local concentration of the Ce^{III}-derived Ce^{IV}/EDTP complex in the present DNA cutters is expected to be large due to the proximity effect. After 20 h in the Ce^{IV}/EDTP solution, 2′-deoxyguanosine, the most readily oxidized naturally occurring nucleoside, remained completely intact (Figure 5b). In striking contrast, after only 30 min in the Fe^{II}/EDTA solution, a significant portion of the same nucleoside was oxidized, yielding guanine and other products (Figure 5c).[11]

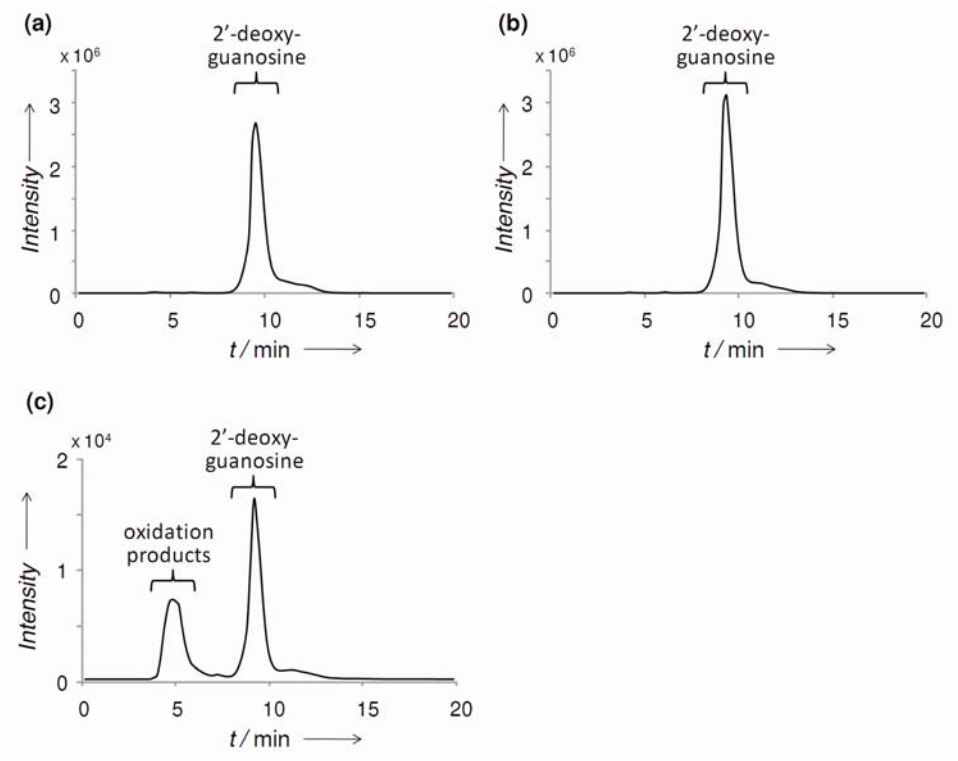

Figure 5. Treatment of 2′-deoxyguanosine by Ce^{III}-derived Ce^{IV}/EDTP complex at 50 °C under air with HPLC analysis of the reaction mixture (a) just after mixing the reagents, and (b) 20 h later. The peaks were detected by UV absorbance at 260 nm. No decomposition of 2′-deoxyguanosine was observed. Reaction conditions: pH = 7.0, $[Ce(NO_3)_3]$ = 5 mM, [EDTP] = 1.25 mM, [2′-deoxyguanosine] = 2 mM, and [HEPES] = 120 mM. In (c), 2′-deoxyguanosine was treated with Fe^{II}/EDTA, which is known to cleave DNA through an oxidative process, at 37°C for 30 min (positive control). The oxidation products including guanine and others were clearly observed at shorter retention times. Reaction conditions; [Fe^{II}/EDTA complex] = 100 μM, [2′-deoxyguanosine] = 100 μM, [H_2O_2] = 10 mM, and [DTT] = 50 μM.

Conclusion

Unprecedentedly well-characterized site-selective DNA cutters have been obtained by oxidation of Ce^{III} ion bound to the oligonucleotide-ligand conjugate. It is noteworthy that the amount of Ce^{III} salt, with respect to the ligand, is far smaller than used in previously reported Ce^{IV} salt-derived DNA cutters. Thus, the amount of unbound catalytic species in the reaction mixtures can be minimized and undesired off-target scission hardly takes place. The present finding should provide a new approach to the design of novel and highly eminent site-selective DNA cutters.

Experimental Section

Commercially available $Ce(NO_3)_3(H_2O)_6$ (from Strem Chemicals Inc.) was used without further purification. EDTP was purchased from Tokyo Chemical Industry Co. The 5'-FAM-labeled substrate ODN and marker ODNs were purchased from Sigma Genosys and purified by usual methods. The oligonucleotide-EDTP conjugates were prepared by the conventional phosphoramidite strategy followed by on-support oxime coupling, as previously described.[8]

For site-selective DNA hydrolysis, the substrate ODN and the additive ODNs were first heated to 95 °C for 2 min and then slowly cooled down to RT. The reaction was initiated by adding a Ce^{III} solution in HEPES buffer (pH 7.0) to the mixture, and carried out at 50 °C under air. After a predetermined time, EDTP was added to a final concentration of 4.5 mM, and the mixture was heat-denatured and immediately subjected to denaturing 20% polyacrylamide gel electrophoresis. The scission fragments were quantified with an imaging analyzer (FLA-3000G; Fuji Film).

For monitoring the oxidation of Ce^{III} to Ce^{IV}, emission spectra were measured on a JASCO FP-6500 spectrofluorometer (λ_{ex} = 313 nm). The Ce^{III}/EDTP solution was prepared immediately before measurement by mixing $Ce(NO_3)_3(H_2O)_6$ in HEPES buffer and EDTP in water (pH 7.0) and then kept under air at 50 °C.

Acknowledgements

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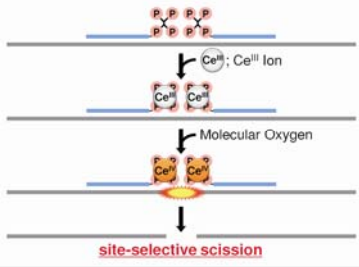

The Ce^{III} complex of a multiphosphonate ligand, bound to an oligodeoxyribonucleotide, was oxidized with molecular oxygen to the catalytically active Ce^{IV} complex, providing unprecedentedly well-characterized site-selective DNA cutter.