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INVITED REVIEW

Artificial site-selective DNA cutters to manipulate single-stranded DNA

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Recent progress regarding the development of artificial site-selective DNA cutters by chemical approaches are reviewed herein, with a special focus on site-selective cutters for single-stranded DNA. We employ a Ce(IV)/EDTA complex to serve as the catalyst, because it efficiently and selectively cuts single-stranded DNA. Using two complementary oligonucleotide additives, a gap structure is formed at the target site in the single-stranded DNA substrate. Owing to the substrate specificity of Ce(IV)/EDTA, the gap site is preferentially hydrolyzed, resulting in a site-selective DNA scission. The scission site is easily determined using the Watson–Crick base-pairing rule; thus, both the sequence and scission specificity can be tuned according to demand. The site-selective scission is greatly promoted by attaching a multiphosphonate to the termini of the oligonucleotide additives and placing this ligand at the gap site. The scission fragments can be connected with foreign DNA using ligase, and the recombinant DNA expresses the corresponding protein in *E. coli*. No undesired side reactions (for example, depurination, deletion, insertion, oxidative damage of nucleobases and off-target scission) occur throughout the DNA treatment.
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INTRODUCTION

The role of DNA in storing and carrying genetic information is essential for the function and development of all living organisms. If the information stored within DNA can be rewritten by scientists, numerous applications in a variety of fields will be possible. On this basis, chemists, biochemists and biologists have long attempted to rewrite the information encoded within DNA using various approaches. One direct and straightforward approach to precisely alter the information contained by DNA is the site-selective scission of DNA at a target site,[1–11] followed by the appropriate manipulation of the DNA at this local site.

Currently, in molecular biology, DNA is manipulated by the 'cut-and-paste' method. In this approach, DNA is first cut at a predetermined site by naturally occurring restriction enzymes, and the resultant fragment is connected with another DNA fragment using ligases. Here, the target gene is inserted, deleted or altered. This strategy is satisfactorily effective so long as small DNAs such as plasmids (composed of several thousand base pairs) are targeted. Unfortunately, this technology is insufficient for manipulating large DNAs; however, our interests have been gradually focusing on the manipulation of genomic DNAs of higher organisms. The key problem is the low site specificity of naturally occurring restriction enzymes (most of them recognize 4 to 8-bp DNA sequences). Statistically, the scission site of a restriction enzyme with 6-bp recognition should appear once every 4096 ($=4^6$) bp. Therefore, if a human genome (3×10^9 bp) is treated with this restriction enzyme, the number of scission sites should be $>7.3 \times 10^5$ ($3 \times 10^9/4096$) on average (see Table 1). Under these conditions, it is impossible to obtain a desired scission fragment and manipulate the genome appropriately. Therefore, artificial DNA cutters (which have higher sequence-specificity and a freely tunable scission site) are absolutely imperative to further developments in biotechnology.

Our group recently developed site-selective cutters that cut DNA via the hydrolysis of a targeted phosphodiester linkage.[12,13] A site-selective cutter for the scission of single-stranded DNA and another cutter for the scission of double-stranded DNA were both prepared.[8,10,11,14–17] Although these cutters are significantly different from each other with respect to their chemical structures, both are primarily based on the substrate specificity of Ce(IV)/EDTA in the catalytic hydrolysis of DNA (only single-stranded DNA is hydrolyzed).[18,19] This article discusses the site-selective cutter for the scission of single-stranded DNA and describes its molecular design and scission specificity. A new DNA cutter that is easily obtainable and useful for the practical manipulation of single-stranded DNA is presented herein. This tool is highly valuable for molecular biology and biotechnology because there are no naturally occurring enzymes that cut single-stranded DNA selectively at a specific sequence. For the purpose of comparison, the site-selective

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Table 1 The number of probable scission sites for the 6-bp recognizing restriction enzyme in various DNA strands

DNA (bp)	*Number of probable scission site*
pBR322 (4361)	$4361/4^6 \fallingdotseq 1$
λ phage DNA (4.85×10^4)	$4.85 \times 10^4 / 4^6 \fallingdotseq 12$
E. coli genomic DNA (4.6×10^6)	$4.6 \times 10^6 / 4^6 \fallingdotseq 1123$
Human genomic DNA (3×10^9)	$3 \times 10^9/4^6 \fallingdotseq 7.3 \times 10^5$

cutter for the scission of double-stranded DNA will be briefly described (those who are interested about these cutters in more detail should refer to recent reviews).[8,10,11,15–17]

THE IMPORTANCE OF ADOPTING A NEW TOOL TO MANIPULATE SINGLE-STRANDED DNA

Single-stranded DNA does not occur as frequently in nature as double-stranded DNA, but the site-selective scission of single-stranded DNA is important for practical applications. For example, the adeno-associated virus (which is currently being utilized for clinical purposes) and some other viruses have a single-stranded genome (approximately 5 kbp).[20–22] If appropriate new tools are available to cut the genome at various predetermined sites, recombinant virus vectors showing unprecedented properties are anticipated to be easily obtained. Furthermore, single-stranded DNA fragments of desired sequences and lengths are readily obtainable by clipping the appropriate segments from naturally occurring DNA sources. These single-stranded fragments should be valuable as building blocks for DNA nanotechnology that is currently in development (DNA nanostructures, DNA origami, molecular machines and so on).[23–32] Other applications for the site-selective scission of single-stranded DNA should also be versatile.

Approximately 30 years ago, synthetic cutters to cleave single-stranded DNA at target sites were developed by several groups. Orgel and coworkers reported that the oligonucleotide bearing Fe(II) complex of ethylenediaminetetraacetic acid (EDTA) at its 5′ terminus cut the target site on complementary DNA.[33] At approximately the same time, the Dervan group also cleaved DNA site-selectively using the Fe(II)/EDTA complex.[34] In addition to the Fe/EDTA complex, another DNA-cleaving agent, the Cu/1,10-phenanthroline complex, was also used to cleave oligonucleotides by both the Sigman[35] and Hélène groups.[36] Although these systems site-selectively cut DNA through an oxidative cleavage of the deoxyribose residues at the target site, the cutters presented in this article cut DNA by hydrolyzing the targeted phosphodiester linkage as naturally occurring nucleases do.[12,13,37,38] The recently reported deoxyribozyme also cuts a single-stranded DNA sequence specifically through a hydrolytic process.[39]

SUBSTRATE SPECIFICITY OF Ce(IV)/EDTA AS MOLECULAR SCISSORS FOR THE PRESENT SITE-SELECTIVE SINGLE-STRANDED DNA CUTTER

In 1994, it was found that Ce(IV) is very effective for DNA scission.[40–42] The DNA scission proceeds completely via a hydrolytic process, and thus, the scission fragments are susceptible to modification by various enzymes (for example, alkaline phosphatase, polynucleotide kinase and terminal deoxynucleotidyl transferase).[12,13] For example, the pseudo-first-order rate constant for the hydrolysis of TpT is 1.9×10^{-1} per hour at pH 7, 50 °C, corresponding to a half-life 3.6 h. Almost a 10^{12}-fold acceleration is achieved, as the half-life of the phosphodiester linkage in DNA in the absence of Ce(IV) is estimated to be 200 million years. The scission rate is hardly dependent on the type of nucleobases adjacent to the scissile phosphodiester linkage. Thus, oligonucleotides are hydrolyzed almost randomly throughout the main-chain. All these features of the Ce(IV) ion are very advantageous for the catalytic center of artificial site-selective DNA cutters.

Ce(IV) is highly susceptible to form a hydroxide gel at pH 7, and this property is inappropriate to design a tool for site-selective DNA scission. Consequently, the Ce(IV)/EDTA complex was developed as a catalyst for DNA hydrolysis.[18,19,43,44] This complex is homogeneous at pH 7 and, more importantly, shows a very unique substrate specificity.[18,19] Thus, the Ce(IV)/EDTA complex efficiently hydrolyzes the phosphodiester bonds in single-stranded DNA, but its catalytic activity for the hydrolysis of double-stranded DNA is minimal. This complex is highly potent as molecular scissors for our targeted site-selective scission of DNA. Only the target site for the scission in the substrate DNA should be kept single-stranded, and the remainder of the DNA should be double-stranded.

This unique substrate specificity of the Ce(IV)/EDTA complex originates from the difference in the stability of the corresponding coordination complexes.[19] The K_m value for the Ce(IV)/EDTA-induced hydrolysis of single-stranded DNA was determined to be 10 μM. On the other hand, according to competition experiments, the K_m value for double-stranded DNA is >5 mM. Therefore, single-stranded DNA binds to Ce(IV)/EDTA >500-fold more strongly than double-stranded DNA and is efficiently hydrolyzed.

FORMATION OF A GAP STRUCTURE AT A TARGET SITE IN SINGLE-STRANDED DNA AND THE SITE-SELECTIVE SCISSION OF THIS SITE BY Ce(IV)/EDTA

The substrate specificity of Ce(IV)/EDTA described above indicates that any target site in a DNA substrate should be selectively cut by this complex, as long as this site is kept single-stranded and the remainder of the DNA is double-stranded (Figure 1).[18,19] To verify this hypothesis, a gap structure was formed at a predetermined site in single-stranded DNA using two complementary oligonucleotides (Figure 2). After treatment with Ce(IV)/EDTA, the product was analyzed by polyacrylamide gel electrophoresis. The scission bands for the site-selective DNA cleavage were weakly but clearly observed (lane 3 in Figure 2b). The sizes of these bands were exactly identical with those derived from the hydrolysis of the phosphodiester bonds at the gap site. Each of the bands corresponds to the scission of the five phosphodiester linkages in the gap structure (the fragments for the scission of one phosphodiester linkage are further separated to two bands depending on whether the 3′-terminus is -OH or -phosphate).

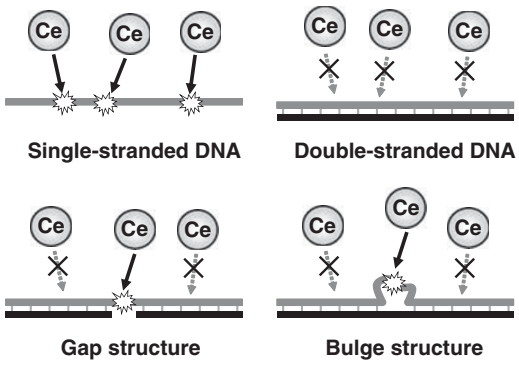

Figure 1 The concepts of gap- or bulge-selective DNA hydrolysis based on the substrate specificity of the Ce(IV)/EDTA complex.

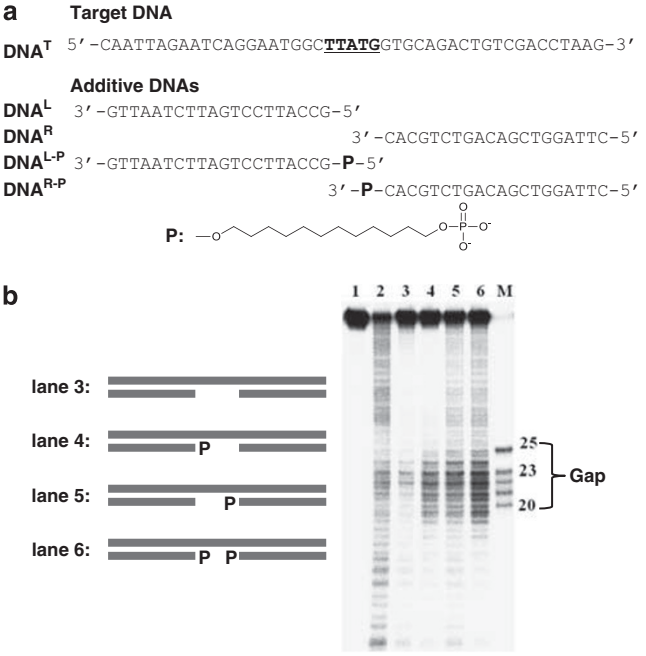

Figure 2 (**a**) Sequences of target DNA and additive oligonucleotides, with or without a terminal monophosphate group, used for the gap-selective DNA hydrolysis. The gap site formed in the substrate DNA^T is shown by the underlined bold characters. (**b**) Typical gel electrophoresis patterns for the site-selective DNA scission by combining Ce(IV)/EDTA with various DNA additives. Lane 1, DNA only; lane 2, DNA with Ce(IV)/EDTA; lane 3, DNA^L/DNA^R with Ce(IV)/EDTA; lane 4, $DNA^{L\text{-}P}$/DNA^R with Ce(IV)/EDTA; lane 5, DNA^L/$DNA^{R\text{-}P}$ with Ce(IV)/EDTA; lane 6, $DNA^{L\text{-}P}$/$DNA^{R\text{-}P}$ with Ce(IV)/EDTA; M, markers. Reaction conditions: $[DNA^T] = 1.0\,\mu M$, [each of the DNA additives] $= 2.0\,\mu M$, [Ce(IV)/EDTA] $= 1.0\,m M$, [HEPES (pH 7.0)] $= 7.5\,m M$ and [NaCl] $= 100\,m M$ at 37 °C for 95 h. Reproduced by permission from the American Chemical Society from Chen *et al.*[46]

Other parts of the substrate DNA forming double-strands with the additives were barely hydrolyzed. Similarly, a bulge-site in the DNA was also selectively cut by Ce(IV)/EDTA (Figure 1; the electrophoresis data are not shown). It is concluded that the single-stranded character at the scission site is critical for the present site-selective scission.

ENHANCING SCISSION EFFICIENCY BY ATTACHING MONOPHOSPHATE AND MULTIPHOSPHONATE GROUPS TO THE OLIGONUCLEOTIDE ADDITIVES

The gap-selective scission of single-stranded DNA by Ce(IV)/EDTA is promoted further by attaching a monophosphate group to the 3′- or 5′-terminus of the oligonucleotide additives (Figure 2).[45,46] When these modified oligonucleotides form duplexes with the substrate DNA, the monophosphate groups at the termini are placed at the edge of the gap site. The site-selective DNA hydrolysis was notably accelerated by introducing a monophosphate (compare lanes 4 and 5 of Figure 2b to lane 3 where no terminal monophosphate was attached). When two monophosphates were placed at both edges of the gap, the site-selective scission was still significantly faster (lane 6). These results indicate that monophosphate groups recruit the Ce(IV)/EDTA to the target site, enhancing both the rate and selectivity of DNA hydrolysis.

It is known that multiphosphonate groups show a higher affinity for lanthanide ions than monophosphate groups. Therefore, in Figure 3, nitrilotris(methylenephosphonic acid) (NTP) and *N,N,N′,N′*-ethylenediaminetetrakis(methylenephosphonic acid) (EDTP) were used, in place of monophosphate (Figure 2), to promote Ce(IV)/EDTA-mediated gap-selective DNA hydrolysis (Figure 3a).[47] In the final stage of the solid-phase synthesis of the oligonucleotides, a protected aminooxy linker **3** was attached to the terminus. This aminooxy group was deprotected and then reacted with 2-(4-formylphenoxy)ethyl esters of NTP and EDTP (**1** and **2**) on the support. As shown in Figure 3b, NTP and EDTP ligands were bound to the oligonucleotides by the formation of oxime. Compared with the primary amino group, the aminooxy group is far more nucleophilic toward the carbonyl group and selectively forms a stable oxime linkage. This reaction proceeds efficiently, even in an aqueous solution, and is well suited for the conjugation of highly hydrophilic NTP and EDTP with oligonucleotides.

It was clearly shown that both the EDTP ligand and NTP ligand at the termini of oligonucleotide additives promote the gap-selective DNA scission effectively.[47] Under the conditions employed in Figure 4, no site-selective DNA scission was observed when unmodified oligonucleotides were used as the additives (lane 1). With two monophosphate groups, the scission was barely observed (lane 2). A single NTP or EDTP at either side of the gap was not very effective (lanes 3, 4, 6 and 7). Only when two of these multiphosphonate ligands (EDTP or NTP) were placed at the gap site, notable DNA scission was accomplished (lanes 5 and 8). Apparently, these multiphosphonate ligands strongly (and probably cooperatively) draw the Ce(IV) species to the gap site, thereby encouraging facile DNA scission. This argument is supported by the plots of DNA scission efficiency vs. Ce(IV)/EDTA in Figure 5. For all systems, the scission efficiency showed a gradual saturation corresponding to the binding of Ce(IV)/EDTA to the ligand-oligonucleotide conjugate. Importantly, the two NTP system saturated at a much smaller Ce(IV)/EDTA concentration than the two-monophosphate system (compare (●) with (▲)). The saturation of the two EDTP system occurred at even smaller Ce(IV)/EDTA concentrations (■).

Note that the present DNA cutting system requires only Watson–Crick base pairing. Thus, the DNA scission occurs efficiently even when the nucleobase(s) at the target site is either methylated or hydroxymethylated (these modifications hardly affect the base-pairing). The applications of this technique to epigenetics are highly promising.

LIGATION OF THE SITE-SELECTIVE SCISSION FRAGMENT FOR THE CONSTRUCTION OF A RECOMBINANT GENE WITH A PRECISE READING FRAME

One of the most important characteristics of the present artificial single-stranded DNA cutter is that it cuts DNA via the hydrolysis of phosphodiester bonds[12,13] exactly as naturally occurring nucleases do. Thus, the scission products by this particular cutter can be a substrate for various enzymatic reactions.[13] This section shows that the DNA fragments obtained by the site-selective scission are actually connected with other fragments using ligase and that appropriate recombinant DNA can be prepared.[48] Furthermore, it is confirmed that the DNAs never suffer from significant side reactions in the course of the scission procedure. In Figure 6, the target gene chosen for manipulation codes for green fluorescent protein (GFP).[49–51] The sense strand of this gene is selectively cut just before the chromophore-coding site, and the resultant upstream fragment is connected with the downstream fragment of the sense strand of blue fluorescent protein (BFP). These two proteins (238 amino acids) are remarkably similar to each other and have almost the same amino-acid sequences, except for three amino acids at the chromophore sites (Cys65, Tyr66 and Thr167 in GFP vs Ser65, His66 and Ile167 in BFP).

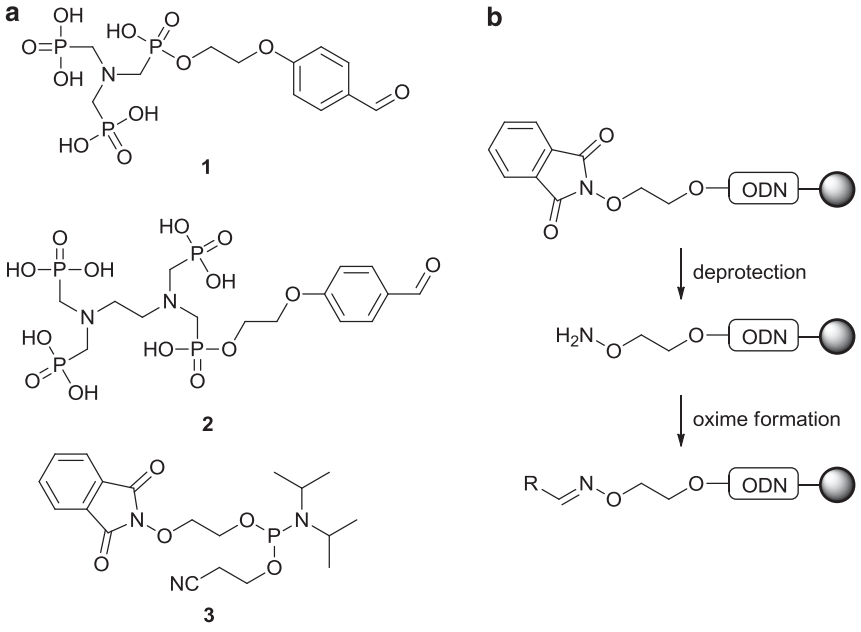

Figure 3 (**a**) 2-(4-Formylphenoxy)ethyl esters of NTP (**1**) and EDTP (**2**) as well as the protected aminooxy linker (**3**) used to incorporate **1** and **2** into the oligonucleotide. (**b**) Preparation of multiphosphonate-oligonucleotide conjugates on the support (R is NTP or EDTP).

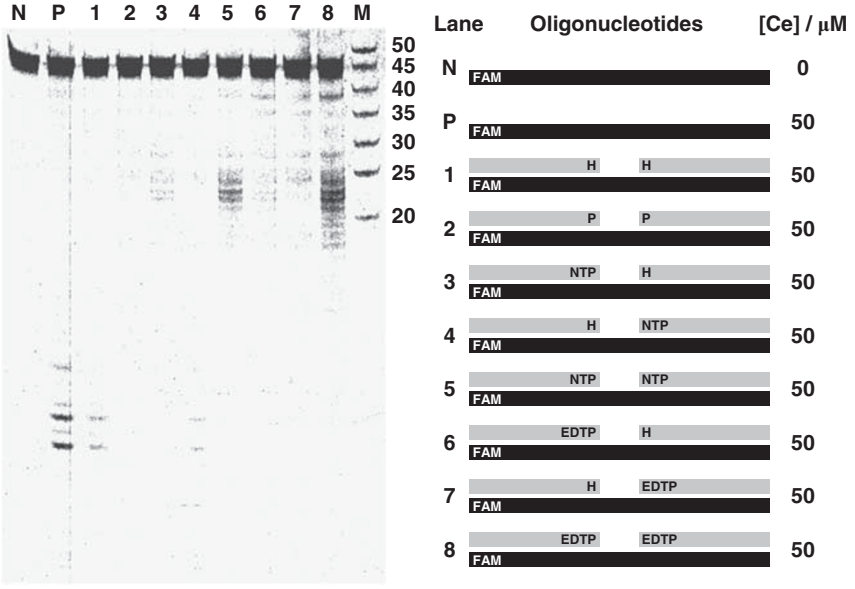

Figure 4 Polyacrylamide gel electrophoresis patterns for the hydrolysis of the target DNA at a five-base gap by Ce(IV)/EDTA. Details of the lanes are presented on the right-hand side. Reaction conditions: [substrate DNA] = 1 μM, [each of the oligonucleotide additives] = 2 μM, [HEPES (pH 7.0)] = 7.5 mM and [NaCl] = 100 mM at 50 °C for 72 h. Reproduced by permission from The Royal Society of Chemistry from Lönnberg *et al.*[47]

Therefore, blue fluorescence should be emitted from the expressed protein if gene recombination from GFP to BFP is successful. Furthermore, the emission wavelength can be a precise measure of how intact the whole gene is during manipulation because even a subtle alteration of the protein structure could induce a notable change in the emission.

An outline of the gene recombination process is given in Figure 6a. The single-stranded DNA substrate (852-mer), involving the whole sense strand of *GFP* gene, was first mixed with two DNA additives that are complementary to A1-G195 and T201-C852, respectively. Upon binding these two DNA additives to the 852-mer GFP-coding DNA, a 5-base gap structure was formed at T196-A200 of the *GFP* gene (a monophosphate group was placed at the edge of this 5-base gap). All portions other than the targeted 5-base gap were double-stranded.

As shown in Figure 6b, the phosphodiester linkages in T196-A200 of the target DNA were selectively hydrolyzed by Ce(IV)/EDTA. After this scission, the upstream scission fragments were purified and recovered by gel purification. The C197-C852 fragment of BFP, which should be ligated with the scission fragments, was prepared

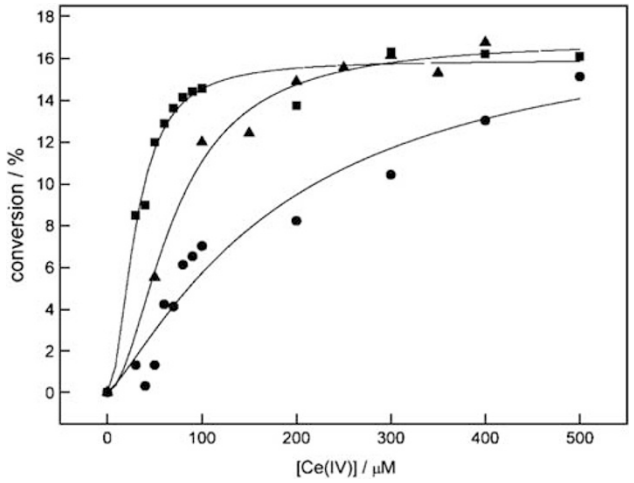

Figure 5 Conversion of gap-selective scission by Ce(IV)/EDTA as a function of Ce(IV)/EDTA concentration. (■) the two EDTP system; (▲) the two NTP system; (●) the two monophosphate system. A pair of these ligands was placed at the edge of the five-base gap, as presented in lanes 8, 5 and 2 in Figure 4, respectively. Reaction conditions: [target DNA] = 1 μM, [each of additives] = 2 μM, [HEPES (pH = 7.0)] = 7.5 mM, [NaCl] = 100 mM at 50 °C for 20 h. Reproduced by permission from The Royal Society of Chemistry from Lönnberg *et al.*[47]

independently. In this experiment, the scission product produced by Ce(IV)/EDTA was a mixture of several fragments that differ in length by several nucleotides, as all of the phosphodiester bonds in the gap site were hydrolyzed by Ce(IV)/EDTA at almost the same rate. To construct the recombinant DNA for successful protein expression, the reading frame of the two fragments must be adjusted correctly. In the present manipulation, for a precise frame adjustment, only one scission fragment of GFP (A1-T196) was selectively picked up from the mixture and allowed to ligate with the downstream of *BFP* gene (C197-C852) (Figure 6c). We found that this task was easily achieved by adding an appropriate template oligonucleotide (20-mer), which binds the two DNA fragments, to the ligation mixture. The template oligonucleotide used in this GFP to BFP conversion is complementary to the 3′-end of the A1-T196 GFP fragment (the green part) and the 5′-end of the C197-C852 fragment of BFP (the blue part) (Figure 6c). Only by adding this template oligonucleotide to the mixture and by performing the ligation as usual, the desired A-T196 fragment was selectively picked up from the mixture of the scission products and ligated to the C197-C852 BFP fragment.

The recombinant DNA was converted to a double-stranded form, integrated into plasmid DNA, and cloned in *E. coli*. Exactly as designed, the protein expressed in *E. coli* emitted blue fluorescence, and its fluorescence spectrum was identical with that of an authentic BFP sample (Figure 7). The sequencing results were also completely consistent with the desired recombinant DNA. Apparently, the recombinant DNAs obtained by the site-selective scission of the present man-made cutter were free from any critical side reactions (for example, depurination, deletion, insertion, oxidative damage of nucleobases, or off-target scission).

NEW SITE-SELECTIVE CUTTER COMPOSED OF TWO MULTIPHOSPHONATE-OLIGONUCLEOTIDE CONJUGATES AND Ce(IV)/EDTA APPLICABLE TO THE PRACTICAL MANIPULATION OF SINGLE-STRANDED DNA

In the previous section, we successfully performed a gene recombination experiment from GFP to BFP. In this system, two long complementary DNAs were used to form duplexes with a single-stranded DNA substrate (with the exception of the targeted gap site) and protect the substrate from any undesired off-target scission. Although this technique could be useful to manipulate single-stranded DNA, the preparation of long single-stranded oligonucleotides is expensive and time-consuming.

To solve this problem and provide a more practical DNA cutter, short EDTP-oligonucleotide conjugates were used to place the EDTP ligands at the gap site. As presented in Figure 4, this multiphosphonate ligand is very effective for promoting site-selective scission by Ce(IV)/EDTA at the gap site.[47] If these short, chemically modified oligonucleotides can be successfully used for the site-selective scission of long, single-stranded DNA strands, the breadth of applications for the cutter as a tool within molecular biology should increase dramatically. In Figure 8, the combination of two short EDTP-oligonucleotide conjugates (the two short gray lines) and Ce(IV)/EDTA was employed to convert the *BFP* gene to a *GFP* gene.[52] An EDTP ligand was attached to the termini of two short oligonucleotides (20- and 21-mer), as shown in Figure 3. These EDTP-oligonucleotide conjugates are complementary with the C225-G244 and the T250-G270 sequences in the sense strand of BFP (838-mer). By mixing these additives and the target DNA, a five-base gap structure was formed between T245 and A249, which is just before (upstream) the chromophore-coding region. The two EDTP ligands were located at the edges of this gap. When this system was treated with Ce(IV)/EDTA, two scission bands (250- and 590-mer) were clearly formed, exactly as expected from the selective scission at the gap site (Figure 8). It should be noted that most of the substrate DNA, on both sides of the gap site (G1-C224 and A271-C838), was single-stranded, but the flanking sections were not hydrolyzed to a measurable extent by Ce(IV)/EDTA; only the gap site was hydrolyzed. Clearly, when two EDTP ligands are placed at the gap site, they strongly attract Ce(IV)/EDTA to the target site; thus, any undesired off-target scission is effectively suppressed.

In the next step, the upstream fragment of the *BFP* gene (G1-T245), obtained by the site-selective scission, was ligated with the downstream fragment of GFP (G246-C838). To the ligation mixture, a template oligonucleotide (20-mer) (which is complementary with the 3′-end of the BFP fragment and 5′-end of the G246-C838 fragment of GFP) was added. Except for the use of this template oligonucleotide, the ligation conditions were identical with those employed in conventional practice. As described in the previous section, simply by adding this template oligonucleotide to the ligation solution, only the A1-T245 fragment of BFP was picked up from the reaction mixture and connected with the downstream fragment of GFP. The reading frame of the resultant recombinant gene was correctly adjusted (see Figure 9a).

Following these procedures, the recombinant single-stranded DNA was converted to a double-stranded form and integrated into plasmid DNA. As shown in Figure 9b, the corresponding protein was successfully expressed in *E. coli* and clearly emitted green fluorescence. The sequencing result confirmed that the G1-T245 fragment of BFP and the G246-C838 of GFP were successfully ligated with a precise reading frame (Figure 9a). Note the nucleotide 211 in the recombinant DNA was T, as established by the sequencing. Thus, this portion was derived from the BFP fragment because the corresponding nucleotide in GFP is G (both CTT211 and CTG211 code Leu). The possibility that the contamination of BFP, from any source, contributes to the observed blue emission is therefore completely ruled out. No mutation was detected except for in the chromophore-coding site. These results further confirmed that this artificial DNA cutter

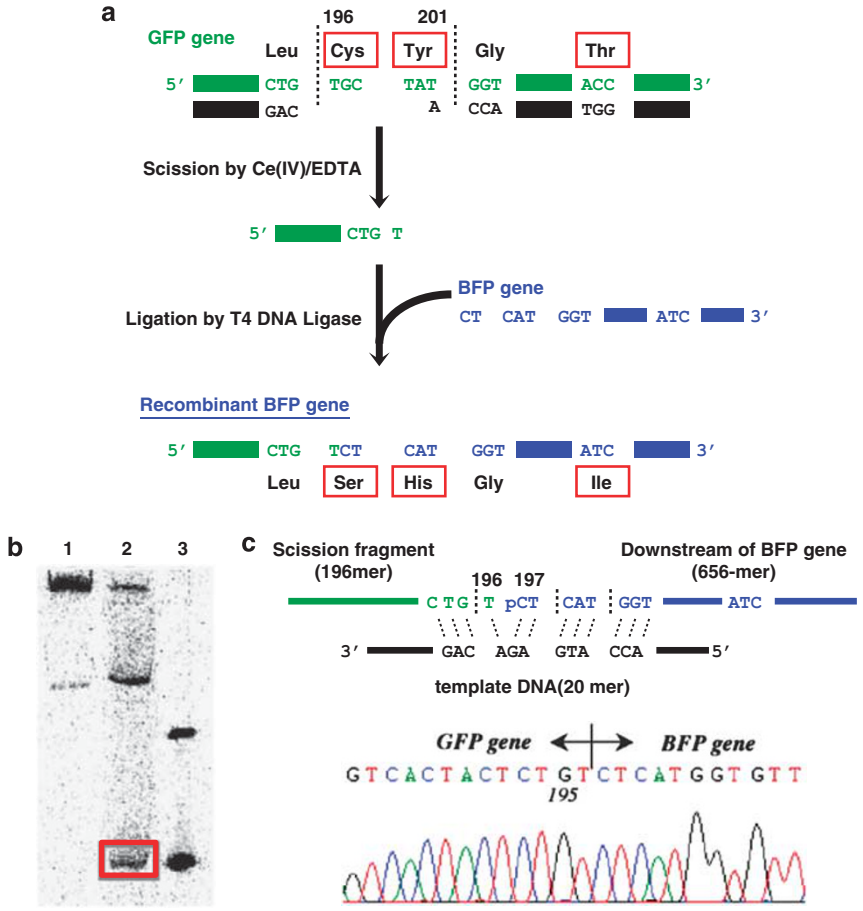

Figure 6 Recombination of GFP to BFP using a man-made cutter of single-stranded DNA in which a five-base gap is formed at the target site and one monophosphate group is placed at this site. (**a**) Schematic depiction of gene manipulation. (**b**) Gel electrophoresis patterns of the site-selective scission of single-stranded DNA (852-mer; the green line in (**a**)). Lane 1, DNA only; lane 2, scission product; lane 3, 195-mer single-stranded and 195-bp double-stranded DNA markers. Reaction conditions: [target DNA] = 0.1 μM, [each of the additive DNAs] = 0.1 μM, [HEPES (pH 7.0)] = 5.0 mM, [NaCl] = 100 mM and [Ce(IV)/EDTA] = 1.0 mM at 37 °C for 40 h. (**c**) Ligation of the scission fragment (in green) with the downstream fragment of BFP (in blue). To pick up the desired scission fragment of GFP (A1-T196) from the scission products, a template oligonucleotide (in black) was used. The sequencing result shows that the desired recombinant DNA was successfully obtained. Reproduced by permission from Springer from Kitamura *et al.*[48]

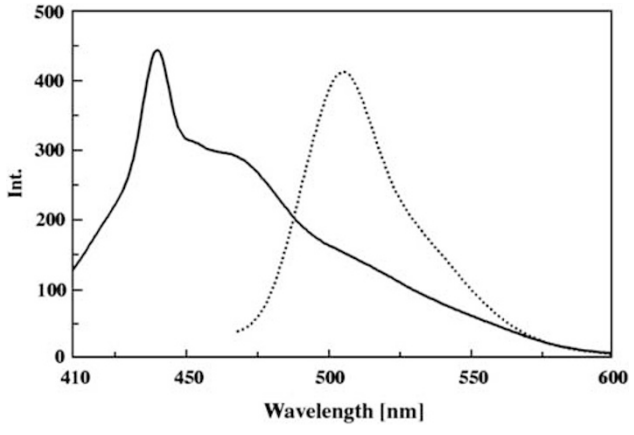

Figure 7 Fluorescence spectrum of the BFP expressed from the recombinant DNA prepared using a man-made artificial cutter of single-stranded DNA (solid line). The details of the recombination procedure are presented in Figure 6. For comparison purposes, the GFP spectrum is also presented (dotted line). Reproduced by permission from Springer from Kitamura *et al.*[48]

does not induce critical side reactions on substrate DNA throughout the manipulation. It should be noted that the EDTP-oligonucleotides (approximately 20-mer) are considerably shorter than the BFP DNA (838-mer), >95% of the target DNA is single stranded, and the target phosphodiester linkages cover only 0.6% of the total phosphodiester bonds in the single-stranded target DNA. Thus, the site selectivity of this system was remarkably high. This strongly indicates that the present cutter (the combination of two short EDTP-oligonucleotide additives and Ce(IV)/EDTA) is applicable for the manipulation of long single-stranded DNA, such as viral genomes.

PREPARATION OF AN ARTIFICIAL DNA CUTTER FROM Ce(III) AS A PRECURSOR FOR CATALYTICALLY ACTIVE Ce(IV)

In the previous section, a new cutter to manipulate a long single-stranded DNA was presented. Undesired scission at off-target sites (the flanking portions which were not protected by duplex formation with the additives) was successfully suppressed by localizing Ce(IV)/EDTA at the target site with the use of the EDTP ligand. However, for more complicated DNA manipulation procedures, further improvements of the site selectivity and a more effective suppression of off-target scissions is desirable. In this section, a second-generation

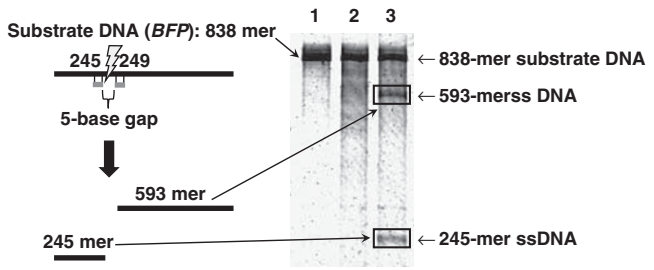

Figure 8 Site-selective scission of a *BFP* gene by combination of Ce(IV)/EDTA with two EDTP-oligonucleotides. Lane 1, DNA only; lane 2, DNA + Ce(IV)/EDTA (without EDTP-oligonucleotides); lane 3, DNA + Ce(IV)/EDTA + EDTP-oligonucleotides. Reaction conditions: [DNA] = 0.1 μM, [each of EDTP-oligonucleotides] = 0.5 μM, [HEPES (pH 7.0)] = 5 mM, [NaCl] = 100 mM, [Ce(IV)/EDTA] = 5 μM at 50 °C for 17 h. Reproduced by permission from The Royal Society of Chemistry from Aiba *et al.*[52]

site-selective DNA cutter is prepared with a Ce(III) salt as a starting material and by attaching a Ce(IV) complex firmly to oligonucleotide additives.[53] Compared with Ce(IV), Ce(III) is markedly less prone to gel formation and more tractable. Although Ce(III) barely hydrolyzes DNA, it can be quickly oxidized by molecular oxygen under neutral conditions and converted to catalytically active Ce(IV).[54,55] These material properties suggest that a well-characterized Ce(IV) complex may be obtained by an *in situ* oxidation procedure if the Ce(III) ions (and the resultant Ce(IV) ions) are sufficiently separated from each other during the oxidation and their mutual aggregation is satisfactorily suppressed.

As the ligand to separate the Ce(III) and Ce(IV) ions from each other, EDTP was employed because it can strongly bind these ions. A site-selective scission of single-stranded DNA was performed as follows. Using two conjugates of EDTP and oligonucleotide (20-mer), a 5-base gap was prepared in the middle of a single-stranded DNA substrate (85-mer). The DNA cleavage reaction was initiated by adding $Ce(NO_3)_3$ to the mixture, and performed at 50 °C for 20 h under air. In the course of the reaction, the Ce(III)/EDTP complex (bound to the oligonucleotides) was rapidly oxidized by ambient

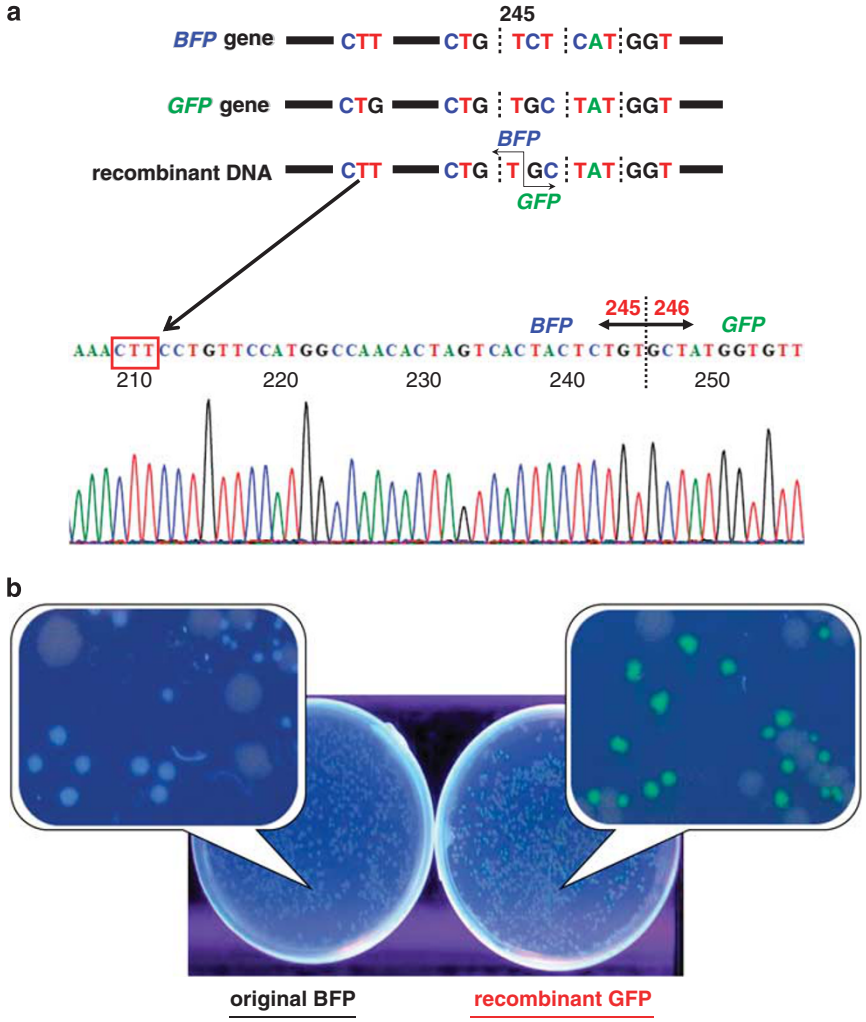

Figure 9 (**a**) The sequencing result of recombinant DNA constructed by the artificial DNA cutter involving short EDTP-oligonucleotide additives. The sequences of the sense strands of BFP and GFP are also shown. (**b**) Expression of the recombinant gene and the original BFP plasmid in *E. coli* (as a control). Reproduced by permission from The Royal Society of Chemistry from Aiba *et al.*[52]

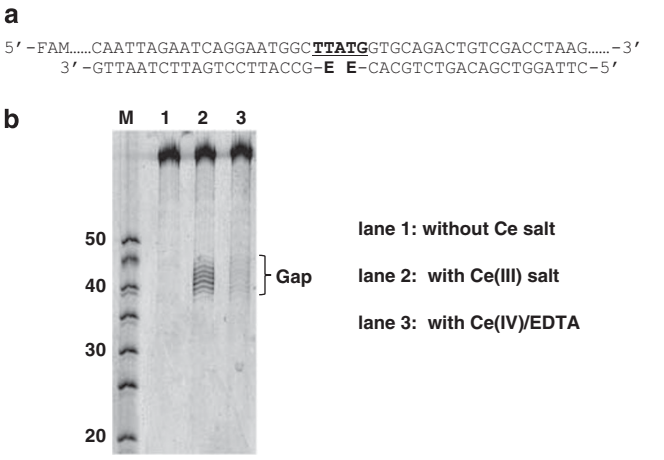

Figure 10 (**a**) The sequences of 5′-FAM-labeled DNA (85-mer) and EDTP-oligonucleotide conjugates (20-mer) used for the site-selective DNA scission by the Ce(III)-derived DNA cutter. 'E' represents the EDTP ligand. (**b**) Denaturing polyacrylamide gel electrophoresis patterns. Lane M, marker; lane 1, target DNA and additive EDTP-oligonucleotides; lane 2, target DNA and additive EDTP-oligonucleotides with $Ce(NO_3)_3$; lane 3, target DNA and additive EDTP-oligonucleotides with Ce(IV)/EDTA. Reaction conditions: [DNA] = 1.0 μM, [each of EDTP-oligonucleotide conjugates] = 1.0 μM, [$Ce(NO_3)_3$ or Ce(IV)/EDTA] = 4.0 μM, [HEPES (pH 7.0)] = 5.0 mM and [NaCl] = 100 mM at 50 °C for 20 h under air.

molecular oxygen to Ce(IV)/EDTP. As shown in lane 2 of Figure 10, site-selective scission occurred at the 5-base gap site, thus producing the corresponding five bands. The DNA scission efficiency increased with an increasing concentration of $Ce(NO_3)_3$ and attained a plateau when the ratio of $Ce(NO_3)_3$ to EDTP was approximately 2-4. These results support that a well-defined Ce(IV) complex on the EDTP ligand is successfully prepared from the Ce(III) precursor, although its stoichiometry has not yet been clearly determined. This new preparation method of a site-selective DNA cutter is advantageous for practical applications because the amount of unbound Ce(IV) is minimized and off-target scission can be successfully avoided.

SITE-SELECTIVE SCISSION OF DOUBLE-STRANDED DNA BY THE COMBINATION OF PSEUDO-COMPLEMENTARY PEPTIDE NUCLEIC ACIDS (pcPNAs) AND Ce(IV)/EDTA

The artificial restriction DNA cutter for the site-selective scission of double-stranded DNA, developed recently by our group, is also based on the substrate specificity of the Ce(IV)/EDTA complex (only single-stranded DNA is efficiently hydrolyzed).[16,56,57] This cutter is composed of two pcPNAs[58–61] and Ce(IV)/EDTA (Figure 11). The pcPNAs used are designed to be laterally shifted to one another by five nucleobases. Thus, when these two pcPNAs invade double-stranded DNA, a gap-like structure (unpaired single-stranded portion) is formed at the predetermined site in each of the two strands of the double-stranded DNA. These single-stranded portions are preferentially hydrolyzed by Ce(IV)/EDTA, leading to the site-selective scission of the double-stranded DNA. With this new tool, various kinds of biotechnological applications, including gene recombination, have been accomplished.[16,57,62–69] Even the whole human genome was selectively cut at one site, opening the way to targeted homologous recombination in human cells.[66,67] The scission efficiency was scarcely affected when the site was methylated.[65]

It is noteworthy that the scission efficiency of this cutter is significantly enhanced by attaching a monophosphonate group to

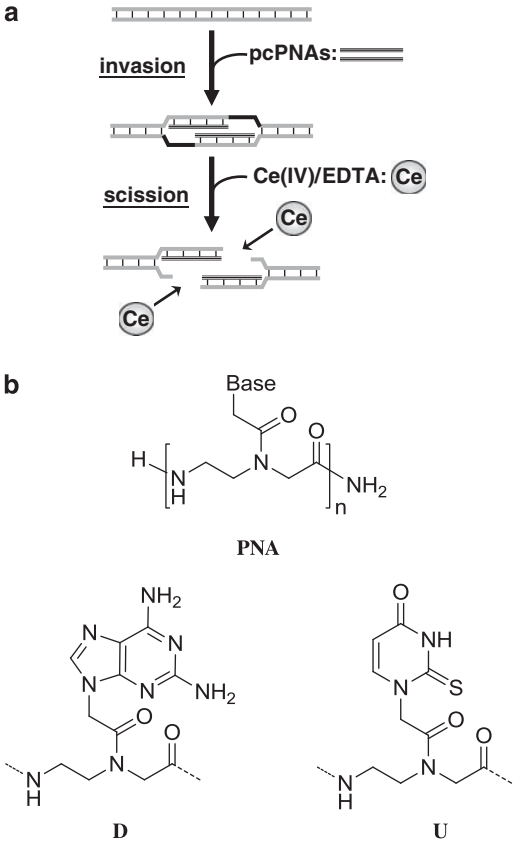

Figure 11 (**a**) Site-selective scission of double-stranded DNA by artificial restriction DNA cutter (ARCUT). Two single-stranded portions (the black lines) are formed by the invasion of pcPNAs and preferentially hydrolyzed by Ce(IV)/EDTA. (**b**) The chemical structures of PNA and the pseudo-complementary bases (2,6-diaminopurine (D) and 2-thiouracil (U)) used for pcPNA. Note that D and U are used simply to promote an invasion process (details are shown in Haaima *et al.*,[59] Lohse *et al.*[60] and Komiyama *et al.*[61]).

pcPNA strands.[65] It has been proposed that these monophosphate groups recruit Ce(IV)/EDTA to the target site, exactly as proposed for the site-selective cutters of single-stranded DNA (Figures 2 and 4). The mechanistic similarity between the single-stranded DNA cutters and the double-stranded DNA cutters is evident. Thus, information to improve the single-stranded DNA cutter can be obtained from a detailed analysis of the double-stranded DNA cutter (and *vice versa*).

As mentioned in the introduction, readers who are interested in more details of artificial restriction DNA cutter and associated work (for example, protein-based DNA cutters) should read the recent reviews and articles on this topic.[8,10,11,15–17,70–72]

CONCLUSION

An artificial cutter for the site-selective hydrolysis of single-stranded DNA has been developed by combining two modified oligonucleotides with Ce(IV)/EDTA. The oligonucleotides bearing an *N,N,N′,N′*-ethylenediaminetetrakis(methylenephosphonic acid) (EDTP) at the terminus are especially effective. No naturally occurring enzymes are available for the site-selective scission of single-stranded DNA, although double-stranded DNA is cut at a specific sequence by restriction enzymes. The site specificity and target site of this artificial DNA cutter can be almost freely modulated; therefore, this cutter should be useful in developing new biotechnology. For instance, viruses with single-stranded genomes can be manipulated to produce

vectors with improved properties. Furthermore, various single-stranded DNA fragments of the desired length and sequence can be clipped from naturally occurring sources and used to construct sophisticated DNA nanomaterials. Even the fragments with various chemical modifications (for example, methylation, hydroxymethylation and non-canonical bases) are obtainable. The cutter described in this study can be, at least in principle, used to manipulate genomes in cells and for other applications *in vivo*. Improvements in the site selectivity and scission efficiency, as well as the fixation of the Ce(IV) complex at the scission site, should further promote its use for various applications. Such attempts are currently underway in our laboratory.

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