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Sensitive voltammetric detection of DNA damage at carbon electrodes using DNA repair enzymes and an electroactive osmium marker

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Abstract This paper presents a new approach to electrochemical sensing of DNA damage, using osmium DNA markers and voltammetric detection at the pyrolytic graphite electrode. The technique is based on enzymatic digestion of DNA with a DNA repair enzyme exonuclease III (exoIII), followed by single-strand (ss) selective DNA modification by a complex of osmium tetroxide with 2,2′-bipyridine. In double-stranded DNA possessing free 3′-ends, the exoIII creates ss regions that can accommodate the electroactive osmium marker. Intensity of the marker signal measured at the pyrolytic graphite electrode responded well to the extent of DNA damage. The technique was successfully applied for the detection of (1) single-strand breaks (ssb) introduced in plasmid DNA by deoxyribonuclease I, and (2) apurinic sites generated in chromosomal calf thymus DNA upon treatment with the alkylating agent dimethyl sulfate. The apurinic sites were converted into the ssb by DNA repair endonuclease activity of the exoIII enzyme. We show that the presented technique is capable of detection of one lesion per approximately 10^5 nucleotides in supercoiled plasmid DNA.

Keywords DNA damage · Strand breaks · Apurinic sites · Electrochemistry · Carbon electrodes · Electroactive marker · Osmium

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Introduction

The main roles of DNA in the cells are to maintain and express genetic information. Damage to the genetic material can arise from interactions of DNA with different chemical or physical agents produced by cellular metabolism or occurring in the environment [1, 2]. When unrepaired, the DNA damage can give rise to mutations and subsequently to severe diseases such as cancer [2]. Analytical methods capable of fast and sensitive detection of DNA damage and/or the DNA-damaging agents are therefore sought. Electroanalytical methods and electrochemical DNA biosensors have successfully been applied in studies of DNA structure and interactions (reviewed in [3, 4]), including applications in sensing DNA damage, DNA–drug interactions, monitoring of genotoxic agents in the environment, toxicity testing, etc. (reviewed in [5–9]).

DNA is an electroactive substance that produces analytically useful signals at mercury, carbon, and some other electrodes, some of whom sensitively respond to changes in the DNA structure [3, 4, 10]. Intrinsic DNA electroactivity and/or surface activity has been utilized in several label-free techniques of DNA damage sensing. For example, a technique of DNA strand break (sb) detection with mercury or amalgam electrodes utilizes a qualitative change in tensammetric responses of DNA upon interruption of the sugar–phosphate backbone of double-stranded (ds) DNA [7, 8, 10–14]. DNA involving the sb is susceptible to unwinding at the negatively charged mercury surface [3, 4, 10]. Thus, part of the nicked (damaged) dsDNA is converted to single-stranded (ss) DNA with freely accessible bases that can be distinguished from (and sensitively determined in the presence of) dsDNA using the mercury electrodes. Intact dsDNA without the sb or free ends of the double helix (such as plasmid supercoiled DNA, scDNA)

cannot undergo extensive unwinding at the electrode surface and its responses at mercury or amalgam electrodes qualitatively differ from responses of the damaged DNA. Under certain conditions it is thus possible to detect a single lesion among approximately 10^5 undamaged nucleotides [11]. Recently we have utilized [13] the same principle, in connection with DNA cleavage by so-called DNA repair enzymes, for a sensitive detection of specific damage to DNA bases.

The most common techniques of electrochemical detection of DNA damage are probably those utilizing a signal due to electrochemical oxidation of guanine (peak G^{ox}) at solid, usually carbon electrodes (reviewed in [5, 7, 8, 15]). Choice of such a detection system is rational because (a) among the DNA constituents, guanine is the most frequent primary target of numerous DNA-damaging agents (including oxidants, alkylating agents etc. [1, 2]) and its chemical modification often results in a loss of its specific electrochemical signal; in addition (b) the relatively cheap carbon electrodes (including disposable screen-printed [9, 16, 17] or pencil lead [18] electrodes) are popular as inexpensive, nontoxic, and widely accessible tools for electrochemical analysis and biosensor development. On the other hand, sensitivity and selectivity of DNA damage detection using the peak G^{ox} is inherently limited by relatively poor sensitivity of this signal to DNA structure changes. It has been shown that intensities of the peak G^{ox} of scDNA and the same but nicked [13, 19] or linearized [19] DNA essentially do not differ which prevents detection of small numbers of sb per DNA molecule. Detection of damage to the guanine bases via measurements of the peak G^{ox} is also limited to rather high DNA damage levels because in this case a decrease of initially large signal is measured. Hence, at least several percent of guanine bases in DNA have to be damaged to obtain a reliable response (depending on reproducibility of the given assay).

In addition to intrinsic oxidation responses of DNA bases ([20]; reviewed in [3, 4]), the carbon electrodes have been applied in connection with various noncovalently interacting or covalently bound electroactive markers [16, 17, 21–27]. Apart from convenient electrochemical properties of the labels (often undergoing reversible redox processes at lower potentials, compared with the irreversible oxidation of the nucleobases), some of them can interact with DNA in a structure-dependent manner. Specifically, an osmium tetroxide complex with 2,2′-bipyridine (Os^{VIII},bipy) forms stable, electrochemically active adducts with pyrimidine bases in DNA. The reaction is strongly selective for thymine residues in ssDNA while modification of thymines in dsDNA is sterically hindered [23, 28–30]. Hence, Os^{VIII},bipy has been utilized as a chemical probe of DNA structure.

Os^{VIII},bipy-modified DNA (DNA-Os^{VIII},bipy) can be determined electrochemically using mercury [28, 30, 31], amalgam [32], gold [24, 33], and carbon [21, 23, 25] electrodes. At the pyrolytic graphite electrode (PGE), DNA-Os^{VIII},bipy produces a specific signal (peak α), the potential of which differs from signals of the free Os^{VIII}, bipy complex [21]. It has been shown that, under certain conditions, subnanogram amounts of Os^{VIII},bipy-modified chromosomal ssDNA can easily be determined at the PGE without purification of the modification products. Osmium DNA markers, in connection with voltammetric measurements at carbon or mercury-based electrodes, have successfully been applied in sequence-specific DNA sensing via DNA hybridization [23, 25, 32, 34].

In this paper we present a new technique for DNA damage detection employing a DNA repair enzyme and the Os^{VIII},bipy electroactive marker. The enzyme is used (a) to create ssDNA regions within dsDNA molecules containing the ssb, and (b) to convert nucleobase damage into single-strand breaks (ssb). The ssDNA regions are modified with the Os^{VIII},bipy marker and the DNA modification is determined by adsorptive transfer stripping (ex situ) square wave voltammetry at the PGE. We show that this approach offers similarly efficient discrimination between intact and subtly damaged DNA as the technique based on utilization AC voltammetry with mercury-based electrodes [11, 12, 35, 36]. Moreover, the presented technique also works well in connection with linear chromosomal DNA (such as calf thymus DNA). Its application is thus not limited to using purified plasmid scDNA.

Experimental

Materials

The plasmid DNA was derived from the single-BbvCI site plasmid described in [37] and hereafter named pL1 (kindly donated by Dr. Darren Gowers, University of Portsmouth, UK). Supercoiled DNA of this pL1 was isolated as described elsewhere [11]. Open (nicked) circular (oc DNA) form of pL1 was prepared by introducing a ssb by BbvC I enzyme [38] (New England BioLabs, Inc.). After the enzymatic cleavage, the DNA was precipitated with ethanol. Single-stranded pL1 DNA was prepared by heating the ocDNA in 10 mM Tris-HCl buffer, pH 7.3, at 100 °C for 6 min followed by rapid cooling in an ice bath. Calf thymus DNA (ctDNA) was isolated as described earlier ([39] and references therein). E. coli exonuclease III (exoIII) was purchased from Takara, pancreatic deoxyribonuclease I (DNase I) from Amersham Live Science. Osmium tetroxide and 2,2′-bipyridine were purchased from

Sigma and dimethyl sulfate from Fluka. Other chemicals were of analytical reagent grade.

DNA digestion with exonuclease III

The DNA samples (30 μg mL^{-1}) were incubated with exoIII in 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM MgCl$_2$, at 37 °C. Enzyme concentrations and reaction times are specified in Results and discussion and/or figure captions. The enzymatic reaction was stopped by addition of Os^{VIII},bipy.

DNA treatment with DNase I

Plasmid scDNA samples (30 μg mL^{-1}) were incubated with DNase I (concentrations specified in figure caption) in the same medium as used for the exoIII treatment, for 30 min at 37 °C. To convert the DNase I-introduced ssb into ssDNA stretches, exoIII (18 U mL^{-1}) was added directly to the reaction mixture.

DNA modification with dimethyl sulfate

Calf thymus DNA (30 μg mL^{-1}) was incubated with the dimethyl sulfate (DMS; concentrations specified in figure caption) in 100 mM Tris, pH 7.3, at 20 °C for 30 min. The samples were then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, using Slide-A-Lyzer MINI Dialysis Units (Pierce).

DNA modification with Os^{VIII},bipy and ex situ voltammetric procedure

DNA samples were treated with 2 mM osmium tetroxide, 2,2′-bipyridine complex (prepared simple by mixing both components at a 1:1 molar ratio) (Os^{VIII},bipy) in 50 mM Tris-HCl, pH 8. The reaction was carried out at 37 °C for 2 h. The modified DNA was adsorbed at the pyrolytic graphite electrode (PGE) directly from the reaction mixture, followed by extraction of unreacted Os^{VIII},bipy from the electrode surface by 30-s washing in acetone [21, 34].

Apparatus

All voltammetric measurements were performed with an Autolab analyzer (Eco Chemie) in connection with VA-Stand 663 (Metrohm). The three-electrode system, involving the basal-plane PGE or hanging mercury drop electrode (HMDE) as working electrode, Ag/AgCl/3 M KCl as reference, and platinum wire as auxiliary electrode, was always used. For the PGE surface renewal/pretreatment, an empirically optimized procedure [21] was used (applying potential +1.7 V for 60 s in the background electrolyte and peeling off the graphite top layer using sticky tape).

Voltammetric measurements

The measurements were done in an ex situ (adsorptive transfer stripping, AdTS) mode [3, 4, 10, 11, 21]. The DNA was adsorbed at the electrode from 3 μL (at HMDE) or 7 μL (at PGE) aliquots at open current circuit for an accumulation time (t_A) of 60 s, followed by washing with deionized water and transfer into the electrochemical cell containing blank background electrolyte in which the measurement was performed. Os^{VIII},bipy-modified DNA was analyzed by square wave voltammetry (SWV) at the PGE using the following parameters: frequency 200 Hz, amplitude 25 mV, scan rate 1 V s^{-1}, initial potential −1.0 V, quiescent time 3 s, positive scan direction (a procedure involving reduction of the osmium species at −1.0 V followed by anodic voltammetric scan, optimized for analysis of osmium-modified DNA previously [21]). An analogous procedure (but with an initial potential +0.1 V) was used for measurements of the guanine oxidation signal (peak G^{ox}). AC voltammetric (ACV) measurements at the HMDE were performed with initial potential −0.6 V, frequency 230 Hz, amplitude 10 mV, scan rate 10 mV s^{-1}, negative scan direction; the in-phase component of the AC current was measured. All measurements were performed at room temperature in air.

Results and discussion

In the label-free voltammetric techniques of DNA damage detection at the mercury or amalgam electrodes, the well-established strong differences between the responses of dsDNA and ssDNA (reviewed in [3, 4, 10]) have been exploited. Segments of ssDNA, adsorbed at the electrode surface via freely accessible bases, yield in AC voltammetry a specific tensammetric signal, peak 3, at a potential of −1.37 V (Fig. 1a, solid curve). The peak 3, albeit smaller, is observed in AC voltammetry at the HMDE or some types of silver amalgam electrodes also with dsDNA possessing free ends (such as open circular DNA, ocDNA; Fig. 1a, dashed curve) due to partial surface denaturation of the double helix during the slow ACV scan [3, 4, 10, 11]. Covalently closed circular scDNA does not yield the peak 3 due to its insusceptibility to the potential-induced surface denaturation (Fig. 1a, dotted curve; the small peak 3 at this voltammogram was due to a small amount of ocDNA present in the scDNA preparation) [11]. Previously we showed that DNA damage involving formation of the sb [11, 12, 14, 19, 35, 39] or nucleobase lesions convertible to the ssb by means of DNA repair endonucleases [13] can be monitored via AC voltammetric measurements of the DNA peak 3. In addition, using E. coli exonuclease III (exoIII)
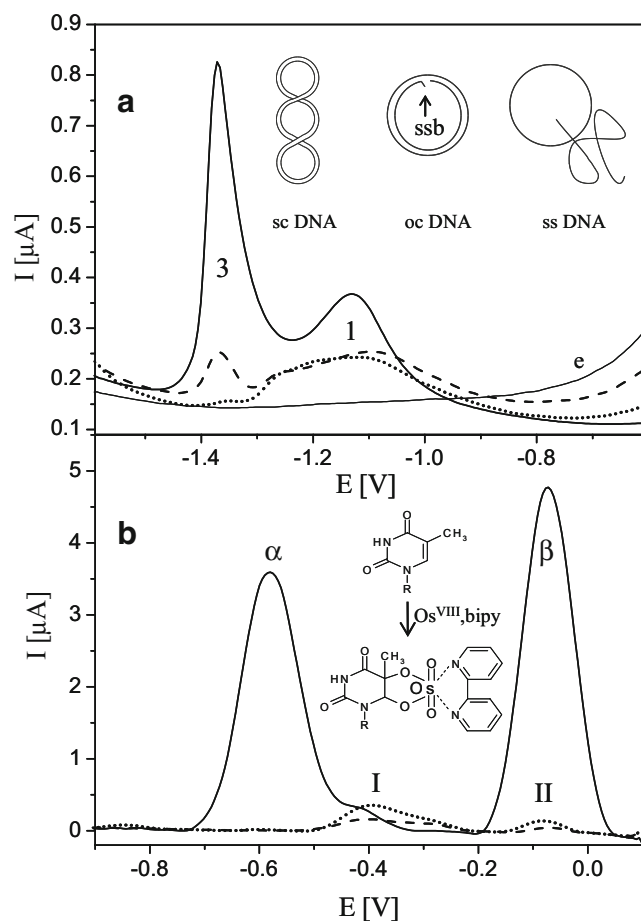
Fig. 1 **a** AdTS ACV responses of the pL1 plasmid DNA at the HMDE: supercoiled (covalently closed circular, *dotted curve*), open circular (containing a single nick, *dashed*) and single-stranded (thermally denatured oc, *solid*). DNA concentration 30 μg mL^{-1}, t_A=60 s, background electrolyte (*e*) 0.3 M NaCl+0.05 M Na$_2$HPO$_4$; initial potential −0.6 V, frequency 230 Hz, amplitude 10 mV, scan rate 10 mV s^{-1}, phase angle 0°, final potential −1.6 V. *Inset* schematic representations of sc, oc, and ss DNA. **b** AdTS SWV responses of the Os^{VIII},bipy-treated pL1 DNA at PGE. Curve styles are assigned to the same DNA forms as in (**a**). The DNA samples (30 μg mL^{-1}) treated with 2 mM Os^{VIII},bipy (2 h at 37 °C) were adsorbed at the electrode surface from the reaction mixture for t_A=60 s, followed by subsequent rinsing with water, ethanol, 30-s washing in acetone, rinsing with ethanol and finally with water. Background electrolyte 0.2 M sodium acetate buffer, pH 5.0; frequency 200 Hz, amplitude 25 mV, scan rate 1 V s^{-1}, initial potential −1.0 V, final potential +0.1 V. *Inset* formation of the thymine–Os^{VIII},bipy adduct

[40], extensive single-stranded regions can be generated in dsDNA possessing free ends or the ssb which has been utilized for amplification of the ssb signal [13].

The same plasmid DNA samples whose AC voltammetric responses at the HMDE are shown in Fig. 1a were treated with 2 mM Os^{VIII},bipy. After 2-h incubation at 37 °C, the DNA-Os^{VIII},bipy was analyzed using a previously introduced AdTS SWV procedure with PGE involving extraction of unreacted Os^{VIII},bipy from the electrode surface with acetone [21, 34]. With ssDNA, two large signals (peak α at −0.59 V and peak β at −0.07 V),

corresponding to reversible electrochemical redox processes undergone by the DNA-bound osmium species [21, 31, 41], were observed with the Os^{VIII},bipy-treated ssDNA (Fig. 1b, solid curve). On the other hand, after the Os^{VIII},bipy treatment both dsDNA forms (scDNA or ocDNA) yielded only negligible peak α specific for the DNA-Os^{VIII},bipy [21] (Fig. 1b, dotted and dashed curves). This observation was in agreement with inaccessibility of the pyrimidine bases in the dsDNA for the modification by the osmium marker[1] [21, 29]. The two small signals observed at −0.40 V and −0.08 V (denoted as peak I and peak II, respectively) have been attributed to small amounts of unreacted Os^{VIII},bipy remaining at the electrode surface after the acetone washing [21].

Taken together, the above results demonstrate that the osmium marker-based technique in connection with AdTS SWV at the PGE offers an excellent discrimination between ss and dsDNA. On the other hand, no significant differences between double-strand DNA forms containing (ocDNA) or lacking (scDNA) the ssb were observed (in contrast to results of the ACV at HMDE, Fig. 1a). Selective conversion of the nicked dsDNA into ssDNA in solution prior to the Os^{VIII},bipy treatment is therefore necessary to render the damaged DNA reactive towards the marker. This can be in principle attained via controlled thermal denaturation of the oc (or linear) DNA in the presence of scDNA that is considerably more resistant towards such treatment [11, 42]. However, this approach cannot be used when damage to linear chromosomal DNA is analyzed (see below). Another possibility is application of the exoIII enzyme that degrades one strand of dsDNA from its 3′-terminus (i.e., from an ssb or from the ends of a linear ds molecule), thus creating an ss region within the dsDNA (Fig. 2). [13, 40]. Undamaged scDNA lacking free ends is not a substrate for the exoIII.

We studied effects of exoIII pretreatment of ocDNA on the peak α intensity measured after DNA modification with Os,bipy. The DNA samples (30 μg mL^{-1}) were incubated with the enzyme (72 U mL^{-1}). Aliquots of the reaction mixture were withdrawn at certain time intervals and mixed with the Os^{VIII},bipy reagent, followed by incubation and AdTS SWV measurements as above. The resulting peak α intensity increased with time of the enzymatic cleavage, reflecting propagation of the ssDNA regions (Fig. 2). The increasing part of the dependence was apparently biphasic, showing a significant increase in slope after 20 min of the

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[0] A closer look into the behavior of sc or ocDNA upon treatment with Os^{VIII},bipy revealed a small but measurable peak α for scDNA but practically no signal for the relaxed ocDNA. This is natural because in the scDNA, the Os,bipy can attack a small portion of pyrimidine bases in regions untwisted due to the negative superhelical stress [30]. Nevertheless, scDNA modification was negligible when compared to the strong modification of the ssDNA.
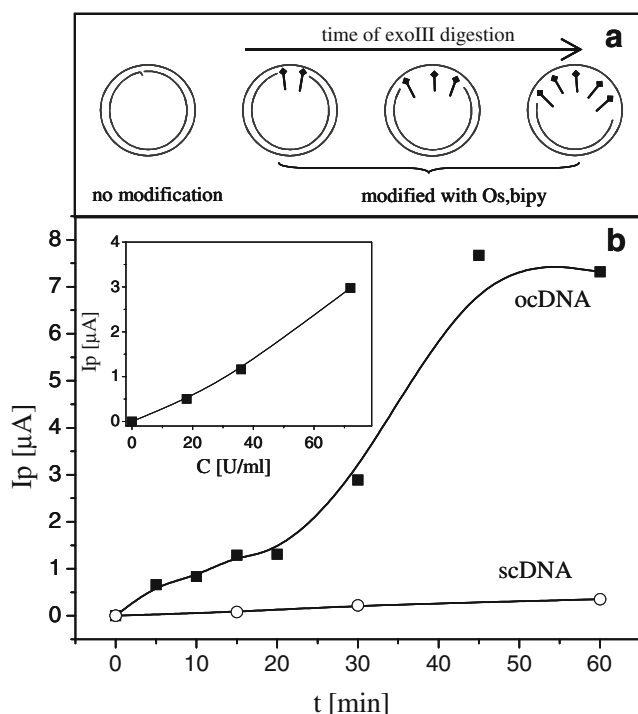
Fig. 2 **a** Scheme of DNA digestion by the exonuclease III (exoIII). The enzyme degrades one strand in dsDNA from its free 3′-end, leaving an ss gap. The ssDNA segments can then accommodate the Os^{VIII},bipy marker. **b** Dependence of peak α heights on time of the DNA digestion with exoIII. Samples (30 μg mL^{-1}) of pL1 oc (■) or scDNA (○) were incubated with exoIII (72 U mL^{-1}) in 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM $MgCl_2$, at 37 °C. *Inset* dependence of the peak α height on concentration of exoIII obtained for ocDNA incubated with the enzyme for 30 min (see Fig. 1 for more details)

exoIII treatment. Such behavior was probably a combination of increasing level of DNA modification with changes in its adsorptivity at the PGE due to gradual loss of ds and gain of ss character of the DNA. Between 45 and 60 min of the enzymatic treatment, no additional increase of the peak α intensity was detected suggesting that the DNA digestion was completed. The inset in Fig. 2 shows the effects of the enzyme concentration on the peak α intensity resulting from 30-min cleavage.

Further, we prepared mixtures of the sc and ocDNA containing 1, 5, 10, and 20% of the oc form (representing in this model experiment damaged DNA in an excess of intact genetic material), and treated them subsequently with exoIII (30 min, 72 U mL^{-1}) and Os^{VIII},bipy (as above). Height of peak 3 increased approximately linearly with the percentage of the nicked ocDNA. A measurable increase of the peak α intensity relative to the background signal (resulting from Os^{VIII},bipy modification of the intact scDNA and/or presence of a small amount of ocDNA in the scDNA sample) was observed even for 1% of ocDNA containing a single nick per molecule. Hence, the high sensitivity towards the ssb (one break in approximately 1% of ca.

3-kb plasmid DNA molecules, i.e., one lesion per approximately 10^5 nucleotides) previously reported for AC voltammetric DNA damage detection at the HMDE [11] can in principle also be attained using the technique presented here. Comparative measurements of the intrinsic DNA peak G^{ox} (without the Os^{VIII},bipy labeling) revealed only a small signal increase (by approximately 15%, in contrast to a ca. 3.5-fold increase of the osmium marker signal obtained for the same DNA sample) even for a 20% content of ocDNA in the plasmid DNA sample (Fig. 3).

Monitoring of DNA single-strand breakage

Strand breaks are amongst the most frequent DNA lesions [2]. The sb can be formed directly due to attacks on the genetic material by some nucleases [43], reactive oxygen species [11, 12], and/or radiomimetic drugs, or indirectly as a consequence of damage to the nucleobase residues (see below). Here we used pancreatic deoxyribonuclease I (DNase I) ([43] and references therein) to generate the ssb. The scDNA was treated with a mix of different concentrations of DNase I and 72 U mL^{-1} of exo III for 30 min, followed by modification with Os^{VIII},bipy and the AdTS SWV measurements at PGE. In agreement with the above data (Figs. 1 and 2), no signal was observed in the absence of the DNase I (i.e., when intact scDNA was treated with exoIII and Os^{VIII},bipy), but appeared when DNase I was added to the cleavage mixture. Intensity of the signal increased with the DNase I concentration between 0 and 0.03 U mL^{-1} and leveled off at higher concentrations of this enzyme (Fig. 4). The ascending part of the dependence
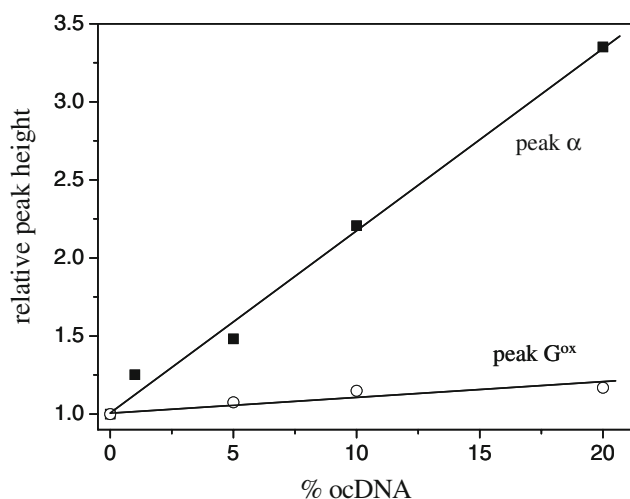

Fig. 3 Effect of relative content of ocDNA in mixture of sc and oc pL1 DNA on the signal intensity: osmium peak α measured after subsequent treatment of the DNA samples with exoIII and Os^{VIII},bipy (■); guanine oxidation peak G^{ox} obtained after the exoIII treatment (○). The signal intensities are plotted as signal-to-background ratio, where background is intensity of the respective peak obtained with control scDNA (0% ocDNA added). Other details as in Fig. 1
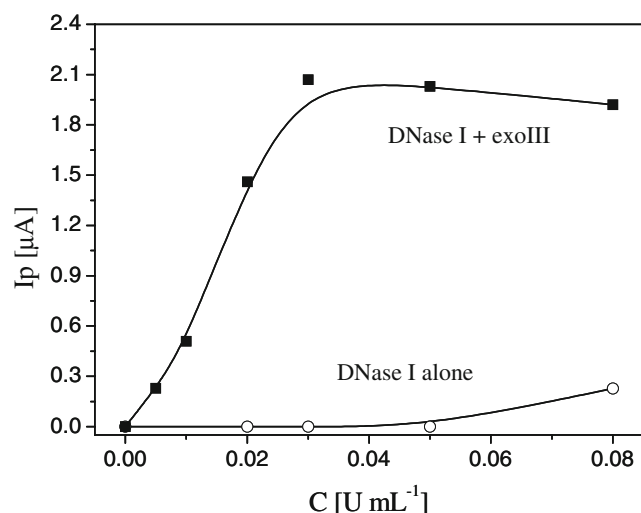
Fig. 4 Detection of ssbs introduced in the pL1 DNA by deoxyribo-nuclease I. scDNA (30 µg mL^{-1}) was incubated with mixtures of DNase I and exoIII (■) or with DNase I alone (○), followed by Os^{VIII}, bipy modification. Dependence of peak α intensity on the DNase I concentration is shown. ExoIII concentration 18 U mL^{-1}, time of enzymatic treatment 30 min; other details as in Fig. 1

reflected an increasing fraction of the nicked DNA mole-cules and/or accumulation of multiple ssb per molecule. Since each ssb represents a starting point for the exoIII cleavage, the proportion of ssDNA formed under the given conditions (and, consequently, the number of Os^{VIII},bipy markers bound) was higher for DNA molecules involving higher number of the ssbs. After reaching a critical frequency (mean mutual distance) of the breaks, the exoIII cleavage can be expected to be completed (for the given cleavage time and enzyme concentration) and the signal to reach its limiting intensity. Such a situation arose for 0.03 U mL^{-1} of the DNase I (Fig. 4). In the absence of exoIII, the scDNA treatment with DNase I at concentrations ≤0.05 U mL^{-1} had no apparent effect on peak α intensity, in agreement with the fact that formation of separated ssbs does not result in denaturation of the DNA double helix. Only after the DNA treatment with rather high DNase I concen-trations (e.g., 0.08 U mL^{-1}, Fig. 4), was a certain increase of the digested DNA reactivity towards Os^{VIII},bipy observed, indicating disintegration of the plasmid DNA molecules into smaller, partially single-stranded fragments due to accumu-lation of many ssb per DNA molecule [35, 43].

Detection of apurinic sites in chromosomal DNA

Damage to DNA bases without interruption of the sugar–phosphate backbone cannot be detected directly by tech-niques sensitive to DNA strand breakage such as relaxation of scDNA detected by agarose gel electrophoresis [11, 43], comet assay [44], or ACV at the mercury-based electrodes [11, 12, 35, 36]. However, the nucleobase lesions can be

converted into the ssb using specific DNA repair endonu-cleases. This approach has successfully been combined with different detection techniques, including those men-tioned above ([13, 45] and references therein).

We used the alkylating agent dimethyl sulfate (DMS) to generate apurinic sites in DNA. In dsDNA, DMS can attack the N7 position of guanine. The resulting N7-methyl guanine is spontaneously released from the nucleotide,
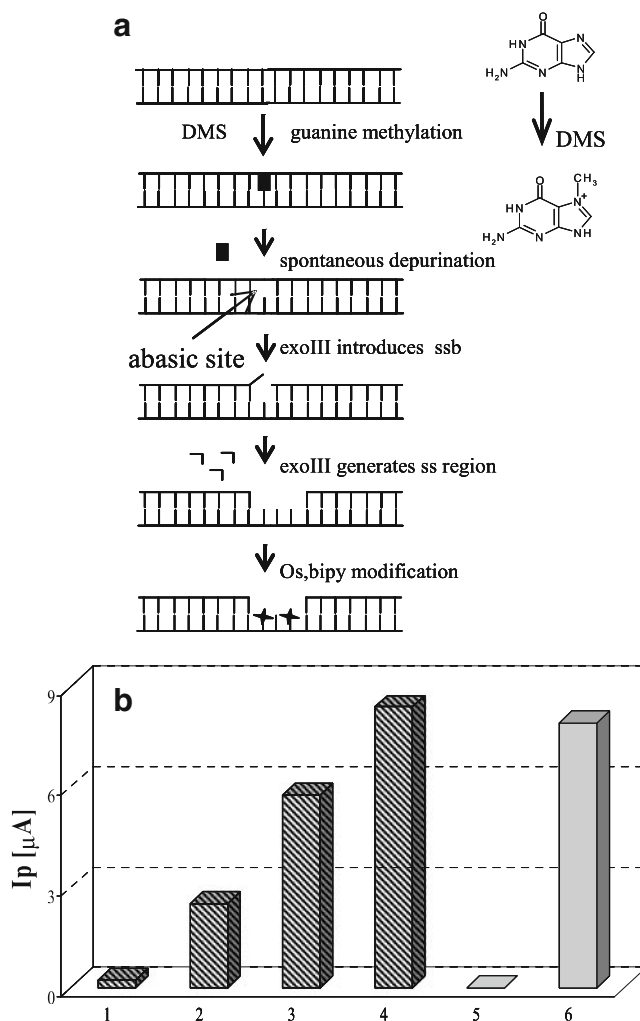

Fig. 5 Detection of abasic sites in chromosomal calf thymus DNA. **a** Scheme of the experiment. Treatment of the DNA with dimethyl sulfate (DMS) results in guanine methylation at the N7-position, followed by spontaneous release of the damaged base. The apurinic sites are recognized by exoIII possessing AP-endonuclease activity that introduces ssbs next to these lesions. The same enzyme then creates ssDNA stretches which are subsequently modified with Os^{VIII}, bipy. **b** Effect of DMS concentration on the peak α intensity (*hatched columns*): 0 (sample *1*), 0.25 (sample *2*), 0.5 (sample *3*), or 1.0 (sample *4*) % (v/v) of DMS. The DNA samples (30 µg mL^{-1}) were incubated with DMS in 100 mM Tris, pH 7.3, at 20 °C for 30 min, followed by dialysis against 50 mM Tris-HCl buffer, pH 8.0. The samples were then treated with exoIII as in Fig. 3. Controls (*grey columns*): DNA damaged by 1% DMS but not treated with exoIII (sample *5*); thermally denatured calf thymus DNA (sample *6*). All samples were modified with Os^{VIII},bipy. For more details see Fig. 1

leaving the depurinated sugar residue (apurinic site) in DNA [1, 13, 46]. The exoIII enzyme exhibits, in addition to the above-mentioned exonuclease activity, a specific DNA repair endonuclease activity, being able to cleave the DNA sugar–phosphate backbone next to the apurinic sites (AP-endonuclease activity) [13, 40]. After introducing the ssb, the same enzyme exonucleolytically degrades one of the damaged dsDNA strands, creating the ssDNA region in the other (see Fig. 5a). Previously we applied [13] this enzyme, in connection with ACV at HMDE or silver amalgam electrode, to detect apurinic sites in DMS-modified scDNA. Here we treated, instead of the plasmid scDNA, chromosomal calf thymus DNA with different concentrations of DMS. The damaged DNA was then incubated with exoIII (18 U mL^{-1}, 30 min) and modified with Os^{VIII},bipy as above. As shown in Fig. 5b, peak α intensities resulting from this experiment increased markedly with increasing dose of the DNA-damaging agent. With undamaged DNA, only relatively small signal was detected after the exoIII cleavage due to formation of ssDNA segments at ends of the linear dsDNA molecules. Since molecules of the chromosomal DNA are relatively long, spanning several tens of thousands of base pairs (provided that the DNA is isolated carefully), relative concentration of their ends is low. In such a situation the background signal of undamaged DNA can be small and the signal increase due to accumulation of the lesions (representing origins of the exonucleolytic digestion by exoIII producing the ssDNA regions that subsequently accommodate the osmium markers) considerable. It should be emphasized that if a poor-quality DNA (involving shorter fragments and/or numerous ssb prior to exposure) were used, the background signal would be higher and detection of low levels of DNA damage more difficult. No peak α was observed with the exoIII-undigested calf thymus DNA even after its treatment with 1% DMS (Fig. 5b).

Conclusions

We have presented a new approach to electrochemical DNA damage detection utilizing a DNA repair enzyme, exonuclease III, to convert single-strand breaks or apurinic lesions into single-stranded DNA stretches. An electroactive marker, Os^{VIII},bipy, is subsequently introduced into the ssDNA regions and the extent of DNA modification is determined by ex situ SWV with the PGE. This approach offers an excellent discrimination between intact and damaged DNA. The osmium marker signal intensity responds to frequency of the DNA lesions. Using plasmid DNA, it is possible to detect one ssb in several percent of DNA molecules, representing one lesion among approximately 10^5 intact nucleotides. Thus, the high sensitivity and

selectivity of electrochemical DNA damage detection, so far attained only with mercury-based electrodes, can be reached via combination of enzymatic approaches and a single-strand selective electroactive marker whose signal is detected with a carbon electrode. Moreover, besides the plasmid scDNA, damage to animal chromosomal DNA can be detected using the proposed method.

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