Redox Labeling of Nucleic Acids for Electrochemical Analysis of Nucleotide Sequences and DNA Damage

Miroslav Fojta

Abstract Electrochemical methods have been established as potent tools to analyze nucleic acids (NA) and their interactions, including DNA or RNA hybridization, DNA-protein interactions, DNA damage, interactions of NA with diverse small molecules including drugs, pollutants and toxins, and so forth. Electrochemical NA sensors have been designed and applied for various purposes related to molecular diagnostics, clinical and environmental analysis, as well as prompt detection of chemical or biological weapons. These devices and techniques may work as label-free ones, utilizing intrinsic electrochemical properties of the NA, or employ electrochemically active and/or enzyme labels to improve their analytical parameters (namely, sensitivity and selectivity). A palette of electrochemically active moieties has been introduced into NA either via chemical modification of natural NA components (such as thymine bases forming redox active adducts upon treatment with osmium tetroxide reagents, or terminal ribose in RNA reacting with six-valent osmium complexes), or via polymerase incorporation of modified nucleotides. The latter technique, utilizing modified deoxynucleotide triphosphates as substrates for the polymerases, represents particularly versatile approach to sequence-specific construction of various modified NA and for multipotential redox coding of nucleobases by organometallics (ferrocene), transition metal chelates or electrochemically reducible or oxidizable organic moieties. A combination of DNA modification with affinity tags (biotin, digoxigenin) with subsequent attachment of enzymes (peroxidase, alkaline phosphatase) has successfully been utilized for signal amplification via biocatalysis.

Keywords DNA labeling · DNA hybridization · DNA damage · Electrochemistry · Biosensors · Multipotential redox coding · Signal amplification

M. Fojta (✉)
Institute of Biophysics Academy of Sciences of the Czech Republic,
Královopolská 135, 612 65 Brno, Czech Republic
e-mail: fojta@ibp.cz

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1 Introduction

Due to their central role in preservation, transfer and expression of genetic information, nucleic acids (NA) belong to most prominent analytes on one hand, or analytical tools on the other, in a broad spectrum of purpose-designed biosensors and bioassays. Based on the detection of specific nucleotide sequences, various organisms, including pathogenic bacteria or viruses, can be identified and detected in biological samples. Application areas of techniques developed to detect nucleic acids sequences (see Sect. 1.1) include those utilized in medicinal diagnostics, in food control (here, detection of transgenes to identify genetically modified crops represents another typical application besides pathogens) as well as detection of biological (bacteriological) weapons (various aspects of these techniquers have recently been reviewed [1–6]). Chemical and/or structural changes in DNA (such as strand breaks, abasic sites or nucleobase adducts) arising from exposure of the genetic material to different environmental or endogenic agents and collectively being referred to as "DNA damage" [7, 8], represent another class of entities for which specific biosensors and bioassay have been designed. A number of genotoxic species interact with (usually double-stranded, ds) DNA non-covalently and in such cases the dsDNA is applied, in various analytical modes, as an affinity substrate to enrich and/or detect the respective substances. Specific class of the NA-based affinity sensors and assays are featured by those employing DNA or RNA aptamers, synthetic in vitro-selected oligonucleotides binding various analytes (from drugs and other small molecules to proteins) with a high selectivity and affinity [9–11]. Unrepaired DNA damage may give rise to mutations i.e., hereditary changes in the genetic information featured by substitution, deletion or insertion of one or more base pairs. Detection of mutations is another application area of DNA sensors and assays with respect to their relation to severe healthy risks. Regardless of the particular application, these approaches can employ various detection platforms (such as optical including fluorescence [12, 13] or surface plasmon resonance (SPR) [4], microgravimetric [14], or electrochemical [2, 5, 6]) and may be designed as either label-free (utilizing solely intrinsic physico-chemical properties of NA or substances interacting with them) or employing various labels or indicators to improve their analytical parameters. This chapter is focused on techniques of NA labeling for the analysis of nucleotide sequences and DNA damage using electrochemical methods.

1.1 Analysis of Nucleotide Sequences

Apart from de novo sequencing of full genomes, nowadays employing fast, high-throughput so called "next generation DNA sequencing methods" developed in the late 1990s [15, 16], the majority of techniques developed for analyzing NA sequences use NA hybridization principle based on the inherent ability of two

complementary NA strands to form the DNA double helix. In the analytical sense, NA hybridization refers to the formation of the duplex NA of a probe (designed to display a specific nucleotide sequence) and a target DNA or RNA strand complementary to the probe [5, 17–19]. The probe can be featured by a piece (oligonucleotide, typically 20–30 base pairs) of DNA, RNA or a synthetic NA analogue (e.g., peptide nucleic acid, PNA [20]; or locked nucleic acid, LNA [21]) developed to improve selectivity of the hybrid formation (such as sensitivity to the presence of base mismatches). Depending on detection platform used in the given assay, the probe or the target can be modified with a suitable label (e.g., radionucleotide, fluorophore, electrochemically active group or affinity tag such as biotin; for more details see Sect. 3). A typical DNA hybridization assay involves immobilization of one of the hybridizing strands (target sequence or the capture probe) on a surface (nitrocellulose membrane as in classical Southern blot assays [22, 23], glass plate as in gene arrays employing fluorescence labeling [13], metal-coated glass prism as in SPR biosensors [4], electrode surface in electrochemical DNA hybridization biosensors [2, 6], or magnetic beads in techniques combining magnetic separation and target enrichment with, in principle, any detection platform [11, 19]). After additional steps typically involving washing and blocking of the surface against non-specific adsorption of the other hybrid-forming strand (signaling probe or target NA) or any other component of the detection system, the hybridization step is performed, followed by another washing cycle and detection. Some detection platforms allow monitoring of all these steps in real time (e.g., SPR [4]).

Another approach of the detection of specific DNA sequence stretches eploys amplification of specific DNA fragments by the polymerase chain reaction (PCR) [24, 25], cyclic DNA replication in vitro. It should be emphasized that the PCR application is essentially based on the DNA hybridization principle as well; in this case, the sequence specificity is rendered by hybridization (annealing) of PCR primers with corresponding sites in the template DNA, delimiting the fragment to be amplified. Amplification products can be detected by various techniques either after running certain number of PCR cycles or in real time (more exactly, cycle by cycle). The real time PCR techniques are well-suited for quantification of the initial concentration of template i.e., specific DNA sequence to be determined in a sample [26, 27]. This technique is frequently utilized for quantifying RNA sequences to determine transcription of specific genes. In this case, RNA sequences are first converted into complementary DNA (cDNA) by RNA-dependent RNA polymerase (reverse transcriptase) which is then subjected to PCR amplification [28, 29].

Besides the detection of a DNA or RNA sequence stretches e.g., to identify and/or detect pathogenic microorganisms, transgenes, or to monitor gene expression, changes in the nucleotide sequences featuring mutations are commonly applied. One group of these techniques applied to detect point mutations (exchange, insertion or deletion of a single base pair) is based on mismatch-sensitive DNA hybridization, utilizing reduced stability of duplexes (heteroduplexes) formed of a wild-type probe and a mutated target (or vice versa) [30, 31]. Under specific conditions, particularly optimized hybridization temperature and medium composition, the heteroduplexes are not stable in contrast to perfectly matched

homoduplexes. In additon, even under less stringent conditions allowing existence of the heteroduplexes, the mismatches can be detected on the basis of altered structure of the DNA double helix at these sites [32, 33] (an example will be discussed in Sect. 3.1). The other group of techniques applied for the detection of mutations is based on polymerase "minisequencing" or "resequencing" of the mutation sites and typically employ labeled nucleosides to incorporate them, on the basis of Watson-Crick base pairing, opposite of the expected site of mutation [34, 35]. Examples will be given in Sect. 3.2.

1.2 Analysis of DNA Damage

The most common products of DNA damage include abasic sites (due to hydrolysis of the N-glycosidic bonds linking the base residue to deoxyribose), strand breaks (sb; i.e., interruptions of the DNA sugar phosphate backbone), 8-oxoguanine (8-OG) arising from oxidative DNA damage or base deamination products (e.g., uracil or hypoxanthine) induced by some known mutagens (such as nitrous acid or bisulfite) [7, 8]. Among others, pyrimidine dimers arise from UV-irradiation of DNA induced by sunlight. Different bulky adducts may be formed due to DNA interactions with metabolically activated carcinogens such as aromatic amines or polycyclic aromatic hydrocarbons. Intrastrand or interstrand cross-links are formed via DNA reactions with bifunctional agents such as antitumor agent cisplatin or a chemical weapon yperite. To analyze damaged DNA and to identify specific products of DNA interactions with the genotoxic species, various analytical techniques were developed. Basically, two groups of techniques can be distinguished. In the first, the analyzed DNA is hydrolyzed into monomeric components (mononucleotides, nucleosides or even free bases, deoxyribose and phosphate) and the DNA damage products are identified and quantified by chromatographic, electrophoretic and/or mass-spectroscopic techniques [36–38]. A variety of DNA adducts have been analyzed by means of so called ^{32}P-postlabeling technique [39, 40] involving adduct-selective enzymatic DNA hydrolysis followed by introduction of ^{32}P radionucleotide, thin layer chromatographic separation and autoradiographic detection.

The other group of techniques is used to measure changes of features of whole DNA molecules upon their damage. Most of these approaches utilize, as the final step, determination of DNA sb by means of gel electrophoresis. Using agarose gel electrophoresis, single (ssb) or double strand breaks (dsb) can be determined with a high sensitivity via relaxation or linearization of supercoiled (sc) plasmid DNA molecules [41, 42]. "Comet" assay is a technique developed to assess the abundance of dsb in genomic DNA of a single cell exposed to DNA damaging conditions [43]. A modification of this technique, alkaline elution assay in which the electrophoresis is run under alkaline denaturing conditions [44, 45], reveals the presence of ssb and alkali-labile sites (together with the dsb), the latter featuring sites of nucleobase lesions. Combination of the electrophoretic methods with DNA

digestion by lesion-specific DNA repair enzymes (N-glycosylases and/or endonucleases) has been employed to detect specifically e.g., abasic lesions, uracil or 8-OG [45, 46]. Abundance of 3‘-OH ends of DNA i.e., strand breaks formed by the action of specific endonucleases (such as those involved in apoptosis or DNA repair) can be estimated by the “TUNEL” test [47], a technique based on labeling of free 3’-OH polynucleotide termini through a reaction catalyzed by a terminal nucleotide transferase. Many of these principles can be combined with other detection techniques, including electrochemical methods.

2 Electrochemical NA Sensing

Among other detection techniques, the electrochemical ones proved a great potential in NA analysis (reviewed e.g., in [2, 48, 49]). Natural NA as well as their synthetic oligonucleotide (ON) analogues possess intrinsic electrochemical activity owing to the presence of nitrogenous nucleobases. Irreversible electrochemical reduction of cytosine and adenine moieties at mercury and silver amalgam [50] electrodes is manifested in a single common signal (peak CA, in random NA sequence). In addition, chemically reversible reduction/oxidation of guanine on mercury and amalgam electrodes gives rise to an anodic peak specific for this base (peak G) [51, 52]. The mercury-containing eletrodes have also been utilized to measure specific tensammetric (capacitive) signals connected with adsorption/desorption (reorientation) processes undergone by the NA at negatively charged electrode surface (reviewed in [2, 49]). The latter signals, as well as the cathodic peak CA, are strongly sensitive to changes in the DNA structure, and have been widely utilized in electrochemical studies of DNA structural transitions and in sensing of DNA damage (reviewed in [7]). All nucleobases have been demonstrated to undergo irreversible electrochemical oxidation on various types of carbon electrodes, and especially the oxidation peak of guanine has found wide application in label-free NA electroanalysis, detection of DNA damage [7], as well as DNA hybridization [6]. Besides the label-free approaches, utilizing solely the electrochemical activity of natural DNA components (albeit sometimes mediated by suitable redox species, such as ruthenium complexes used for catalytical oxidation of guanine residues [53]), a number of techniques based on applications of redox labels or indicators or labels have been introduced [54–57]. Techniques of electrochemical DNA labeling and their applications are reviewed in Sect. 3.

2.1 Adsorptive Stripping of NA and NA-Modified Electrodes

Electrochemical analysis of nucleic acids usually utilizes stripping techniques to reach a high sensitivity of NA detection by adsorptive preconcentration at the electrode surface. Owing to a high adsorptivity of NA and their analogues on

mercury, amalgam or carbon electrodes, a high surface concentration of the NA can be achieved after a short accumulation time (one or several minutes) even when its solution concentration is low [49]. Moreover, adsorption of NA at the above mentioned electrodes is firm enough to resist medium exchange [2, 58]. Thus, NA can be adsorbed at the electrode from a small (several microliters) drop of sample solution to create a NA-modified electrode. Responses of the adsorbed NA are then measured in a usual electrochemical cell in blank (lacking any NA) background electrolyte. This procedure, called adsorptive transfer stripping (AdTS), medium exchange or ex situ electrochemical analysis, has extended application possibilities of nucleic acids electrochemistry: (i), low requirements of the sample volume has made AdTS procedures suitable for analyzing samples that are usually not available in large quantities, including sample series in typical magnetic beads-based NA assays (see below); (ii) some limitations inherently connected with the conventional electrochemical measurements (such as the necessity of dissolving the analyte in media in which the given electrode process can take place) are circumvented via the medium exchange; (iii) an electrode with adsorbed NA layer can be used as a simple electrochemical biosensor. DNA modified mercury, amalgam or carbon electrodes have successfully been applied as biosensors for DNA damage [7, 42]. In the area of DNA hybridization sensors, carbon electrodes with probe or target DNA simply adsorbed onto the electrode surface have been successfully applied [28, 59, 60] in addition to other solid electrodes modified with covalently immobilized capture probes (e.g., thiolated ONs chemisorbed on gold or carbon electrodes with ON probes attached via carbodiimine chemistry [2, 61, 62]).

2.2 *Techniques Combining Separation on Magnetic Beads with Electrochemical Microanalysis*

Magnetic beads (MB) modified with various biomolecules (nucleic acids, streptavidine, antibody-binding proteins, antibodies etc.) are commercially available tools frequently used for convenient affinity capturing, enrichment and isolation of specific biomolecules from complex matrices (such as biological samples) [11, 19]. Among other applications, the MB have been applied in techniques developed for detecting DNA hybridization [18, 63–65], DNA-protein interactions [33, 66–68], studies of chemical modification of DNA [69] or preparative strand separation [35, 70, 71]. These procedures can conveniently be connected with AdTS electrochemical analysis. Compared to "classical" concepts of electrochemical DNA sensors, using the same (electrode) surface for probe immobilization, binding of the affinity partner and detection, the MB-based techniques have been named by Palecek et al. "double-surface" i.e., using two different surfaces for interaction and detection [19]. The latter concept allows in principle much easier optimization of both steps because, being spatially separated, they do not affect one another. The carrier surface need not be electrically conductive and possess other properties critical for analytically applicable working electrodes. On the other hand, the

detection electrode and electrochemical technique can be chosen only with respect to the particular analyte and given electrode process: one electrode type may be best suited for label-free NA detection while another for determination of e.g., modified signaling probes labeled with electrochemically active tags or for detection of electroactive indicators generated in techniques employing enzyme labels (see below). In such applications, the working electrodes do not require specific laborious pretreatment and can include any of the electrodes suitable for AdTS electrochemical analysis of NA or simple organic compounds generated as indicator products in enzyme-linked techniques (e.g., mercury, silver amalgam and various types of carbon electrodes, such as pyrolytic graphite, carbon paste, pencil graphite, or screen printed electrodes).

3 Labeling of NA for Electrochemical Sensing and Its Applications

To improve sensitivity and selectivity of electrochemical analysis of NA, various extrinsic electroactive species have been applied. One group of such species include soluble redox indicators that selectively interact with dsDNA non-covalently, typically via intercalative or groove binding [54, 55] and provide discrimination between duplex (target-probe hybrid or intact dsDNA) and single stranded (probe or target alone) or damaged DNA. Another group includes covalently bound redox moieties that are used to label a nucleotide sequence (hybridization signaling probe [72] or a sequence-specific DNA target for DNA-protein interaction studies [67]), or even to encode a specific nucleotide (e.g., for the detection of point mutations or single nucleotide polymorphisms). The following sections are devoted to techniques of covalent redox labeling of NA and their selected applications.

3.1 Modification of Nucleic Acids with Oxoosmium Complexes

3.1.1 Modification of Nucleobases with Osmium Tetroxide Reagents

Osmium tetroxide reacts with alkenes to give vicinal diols in a reaction involving [3 + 2] addition of osmium tetroxide on the C = C double bond giving rise to an osmic acid diester glycolate, that is subsequently hydrolyzed to corresponding glycol moiety and osmate (reviewed in [56]). Analogous reactions are given by various compounds possessing the C = C double bonds, including pyrimidine nucleobases (at C5 = C6). It has been further shown that tertiary amines such as 2,2'-bipyridine (bipy), 1,10-phenanthroline (phen) derivatives, or N,N,N',N'-tetramethyl ethylenediamine (TEMED), stabilize the osmate glycolates due to coordination of the central osmium atom by the nitrogenous ligands. Products of

modification of the pyrimidines with osmium tetroxide in the presence of the nitrogenous ligands are stable adducts retaining the osmium moiety and the given ligand (Fig. 1a). In contrast, nucleobases do not react with analogous oxo-osmium (VI) complexes (see Sect. 3.1.2) under anaerobic conditions [73, 74]; however, in the presence of ambient oxygen or other oxidants, such as hexacyanoferrate(III) or N-methylmorpholine N-oxide [75], the above mentioned products of reactions at the C = C double bonds are formed.

In nucleic acids, reactions with the osmium tetroxide complexes (further referred to as Os(VIII),L) are strongly selective for pyrimidine nucleobases (reviewed in

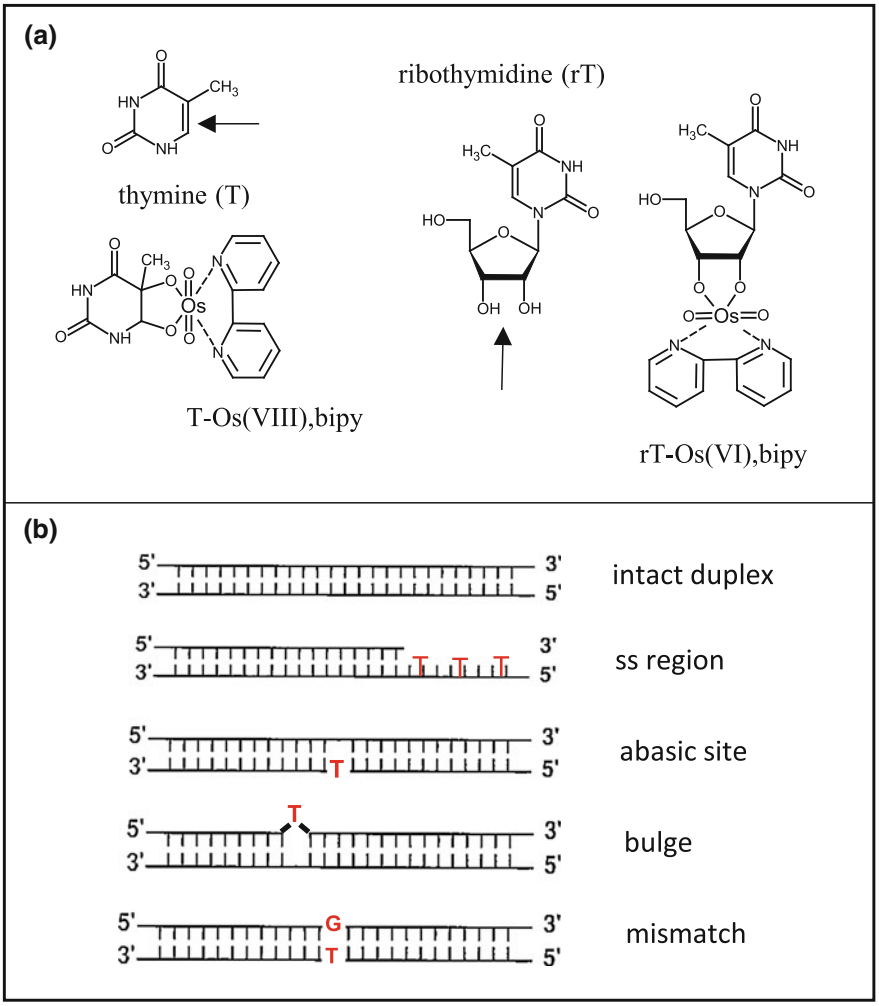

Fig. 1 Modification of nucleic acids with oxoosmium complexes. **a** Reaction products of Os (VIII),bipy with thymine nucleobase and of Os(VI),bipy with ribose. **b** Schematic representation of sites in DNA comprising thymine residues reactive to Os(VIII),bipy (in *red*)

[56]). Remarkable differences in the reactions rates with OsO4 or Os(VIII),L have been observed among natural pyrimidines differing in substituents at position 4 and 5. In general, keto group at C4 and methyl group at C5 facilitate modification at C5 = C6 of thymine (4-keto, 5-methyl) modification proceeds by an order of magnitude faster, compared to modification of cytosine (4-amino, no methyl), while reaction rates observed for uracil (4-keto, no methyl) and 5-methylcytosine (4-amino, 5-methyl) are intermediate [56, 76].

The DNA-Os,L adducts exhibit distinct electrochemistry due to the presence of the osmium central atom which gives several analytically useful faradaic signals due to consecutive redox processes [77]. Moreover, the final reduction step at mercury electrodes is coupled to catalytic hydrogen evolution [77] allowing determination of low concentrations of the osmium-labeled DNA (or low levels of DNA modification). Besides the mercury drop electrodes, the osmium catalytic processes were also observed at glassy carbon electrodes covered with mercury film [78] and at solid silver (AgSAE) or copper amalgam electrodes modified with mercury meniscus [79]. At carbon electrodes, faradaic current signals due to reversible redox processes of the osmium moiety can be measured [63, 72, 80]. Redox potentials of the DNA-Os,bipy adducts differ from those of the free Os(VIII),bipy reagent, which in principle allows to determine the DNA-Os,bipy in the presence of unbound complex. Further, the redox potentials of the osmium DNA markers depend on the nitrogenous ligands, making it possible to use different ligands for "multicolor" (multipotential) DNA coding [72]. The faradaic responses of the osmium-modified DNA have also been measured with gold electrodes [81–85].

Applications related to DNA hybridization. Os(VIII),L modification of signalling (reporter) probes or target DNAs has been introduced as a convenient approach to analyzing DNA sequences via DNA hybridization [63]. ON probes comprising a 5'- or 3'-terminal oligo(T) tail attached to a recognition sequence have been designed, in which the osmium labels were naturally accumulated in the oligo(T) tail. Using longer tails (20–30 thymines) adopting corresponding number of the osmium labels it has been possible to reach remarkable signal amplification resulting in low detection limits. To obtain an osmium-labeled RP capable of hybridization (i.e. with intact recognition sequence), modification of nucleobases within the probe recognition segment has to be precluded. When the probe contains a homopurine recognition stretch and the oligo(T) tail, it can be safely treated with Os(VIII),L in its ss form thanks to a highly preferential modification of the thymine residues and very low reactivity of purine bases towards the osmium reagent [63, 72]. Probes possessing cytosine (but not thymine) residues within the recognition sequence can be modified without losing their hybridization capacity using milder reaction conditions. However, in the case of probes designed for hybridization with target DNA sequences involving all four nucleobases, the recognition stretch of the probe must be protected during the modification reaction by "protective strands"—ON complementary to the probe recognition sequence. Then only thymine residues in the oligo(T) tail (forming a ss overhang) are exposed to the reagent, while those within the specific sequence are hidden within duplex DNA and do not react with Os(VIII),L. Separation of the labeled probe strand from a terminally biotinylated

protective ON can be attained using MB covered with streptavidine [56]. In this way, signalling probes labeled with various Os(VIII),L reagents (differing in the ligand L to "tune" redox potentials of the probes) were prepared and applied in parallel analysis of multiple target DNA sequences. An alternative approach developed by Flechsig et al. [84] has been based on the application of protective strands involving several mismatches with respect to the recognition sequence in a target DNA. Due to these mismatches, strand exchange between the protective oligonucleotide and the fully complementary capture probe (attached via thiol linkage to a gold electrode) is favored towards formation of the more stable fully matched hybrid and thus the extra strand separation step can be omitted. The latter technology has been used in a variety of applications to detect synthetic as well as natural PCR-amplified target DNAs [82, 83, 85–87], including sequences related to transgenes present in genetically modified crops [87].

Applications related to DNA damage. As mentioned above, modification of thymine residues with Os(VIII),bipy in dsDNA (where they are base-paired with opposite adenines) is precluded, in contrast to facile modification of unpaired thymines in ssDNA (Fig. 1b). Hence, DNA lesions resulting in disruption of the base pairs, as well as those convertible into ssDNA, can in principle by detected via chemical probing with Os(VIII),bipy to improve structure selectivity and sensitivity of electrochemical DNA damage detection. For example, label-free detection of DNA strand breakage via measurements of guanine oxidation signal at carbon electrodes is inherently much less sensitive, compared to label-free determination of DNA strand breaks with mercury electrodes [7]. While down to one break per 10^5 nucleotides (i.e., a ssb in one per cent of plasmid DNA molecules) can easily be detected by voltammetry at mercury electrodes [42], using carbon electrodes (which are otherwise better suited for applications in biosensing due to their non-toxicity, mechanical stability, disposability and low cost) it is practically possible to detect a ssb per one plasmid DNA molecule [88]. Nevertheless, combination of DNA treatment with E. coli exonuclease III (EXOIII) to generate terminal or internal ss regions in dsDNA and their subsequent Os(VIII),bipy modification has provided a highly sensitive detection of DNA damage with carbon electrodes [89]. Covalently closed circular duplex DNA molecules lacking any ends are not substrate for the EXOIII enzyme. Once the DNA molecule contains a ssb, the enzyme degrades one of the dsDNA strands from its 3'-OH terminus, generating ssDNA stretch in the other strand which then accumulates the osmium tags. Other types of lesions can be detected in this way as well after their conversion to the strand breaks by suitable DNA repair glycosylases and/or nucleases.

A basic lesions featured in sites of missing adenine residues, leaving opposite thymines unpaired, represent another class of entities reactive towards Os(VIII), bipy [90] (Fig. 1b). It was demonstrated that, besides simple direct voltammetric determination of the DNA-bound osmium, also indirect immunochemical approaches can be applied. Binding of an anti-DNA-Os,bipy antibody to the osmium-DNA adducts can be monitored either via an enzyme-linked electrochemical assay (see Sect. 4 for more details), or via acoustic thickness shear mode detection. Similarly, thymines in single-nucleotide bulges (resulting from insertion

mutations) or base mismatches (resulting from single base substitutions) can easily be detected through introduction of the osmium marker at the unpaired or mispaired thymine followed by AdTS voltammetry at the pyrolytic graphite electrode [32].

3.1.2 Modification of Terminal Sugar Residues in RNA with Six-Valent Osmium Complexes

In general, vicinal diols, including those present in carbohydrate moieties, undergo condensation reactions with six-valent osmium (osmate) complexes Os(VI),L, yielding osmate glycolates [91] (Fig. 1a). The Os(VI),L readily react with cis-1,2-diols, and usually do not react with trans-1,2-diols; nevertheless, exceptions from this rule were identified for e.g., some glucose derivatives. Application of Os (VI),L modification to introduce redox labels into oligo- and polysaccharides [92, 93], as well as glycan components of glycoproteins [94] were reported. Attention has been also paid to modification of ribose moieties in ribonucleosides and ribonucleotides [74, 95, 96]. $K_2OsO_2(OH)_4$, in the presence of suitable nitrogenous ligands, reacted with the sugar residue of all usual ribonucleotides. Os(VI),bipy showed specific modification of ribose in thymine and adenine ribosides in comparison with unreactive deoxynucleosides; no modification of thymine base with the six-valent osmium was observed under anaerobic conditions. In contrast, Os(VIII),bipy reagent modified only the thymine base; the two reagents thus offer exclusive modification of NA components on either ribose sugar, or on pyrimidine nucleobase [74]. Specific 3‘-OH terminal labeling with Os(VI),bipy has been utilized in the development of MB-based electrochemical techniques for the detection of microRNAs, important biomarkers related to severe diseases including cancer [17, 97].

3.2 Polymerase Incorporation of Redox-Labeled Nucleotides

A potent and highly versatile approach to preparation of labeled (or, generally, chemically functionalized) nucleic acids consists in the incorporation of modified nucleotides into NA (ON) by polymerase enzymes, using corresponding modified deoxynucleotide triphosphates (dNTP) as precursors (reviewed in [57, 98]). The external conjugate groups may be attached to the nucleotides on sugar moieties or on nucleobases, and may comprise fluorophores [99], chemically reactive groups for further conjugation reactions [100–102], bioaffinity tags (biotin or digoxigenin) [28, 34] as well as electrochemically (redox) active moieties [55, 57]. While some of the modified dNTPs are commercially available (e.g., those labeled with fluorescein, biotin, digoxigenin, electroactive ferrocene or azide for subsequent coupling reactions via click chemistry), development of new types of labels and labeling approaches represents a dynamically developing field of NA research.

Development of electrochemically labeled dNTPs in recent years opened new possibilities in electrochemical NA sensing. Incorporation of the labeled nucleotides into DNA is potentially useful not only for preparation of labeled hybridization probes, but also for approaches based on sequence-specific introduction of the electroactive tags (e.g., detection of expected mutations via DNA minisequencing [35, 71, 103]). Moreover, application potential of these techniques can be further extended by application of electrochemically or chemically switchable labels [104, 105] or by ratiometric analytical approaches [70], as presented and discussed in the following paragraphs.

3.2.1 Base-Modified Redox-Labeled dNTPs

Recently developed facile synthesis of base-modified dNTPs using aqueous cross-coupling reactions (reviewed in [98]) has resulted in considerable extension of the portfolio of redox-labeled nucleotides. Depending on the nature of the external conjugate groups and related reaction conditions, either direct cross-coupling of these moieties to dNTPs, or modification of nucleosides followed by their triphosphorylation, have been applied. With respect to compatibility of base modifications with subsequent polymerase reactions (see Sect. 3.2.2), either pyrimidines substituted at position 5, or 7-deazapurines substituted at position 7 have been selected for these studies (Fig. 2) (C8-substituted purine dNTPs are not accepted by the polymerases as useful substrates). The dNTP modifications are typically prepared via Sonogashira cross coupling reaction of corresponding iodinated nucleosides or dNTPs and ethynyl derivatives of the label to be attached, or via Suzuki-Miyaura reactions of the iodinated precursors with arylboronic acids.

Redox active moieties coupled to the dNTPs in this way include "classical" reversible redox systems such as ferrocene, tris-bipyridine complexes of $Os^{3+/2+}$ or $Ru^{3+/2+}$ (in which the redox electrochemistry is rendered by the metal component) [35] or athraquinone (quinone/hydroquinone redox couple) [104]. Another group of newly developed redox labels include organic nitrogenous and/or oxygenous moieties which are electrochemically reducible (e.g., nitroderivatives [71], benzofurazane [70], hydrazones [100, 106, 107], aldehydes [100], azidophenyl [105]) or oxidizable (e.g., aminoderivatives [71], methoxyphenol or dihydrobenzofuran [108]). Albeit electrochemical processes and signals yielded by these substances are usually irreversible, some of them exhibit other convenient properties such as high electron yields upon their electrochemical transformation, offering a high sensitivity of detection (e.g., the nitro group undergoes four-electron reduction on mercury and carbon electrodes, while benzofurazane is reduced with six electrons on merury and apparently with four electrons on carbon; both labels give very well developed and analytically useful signals [70]).

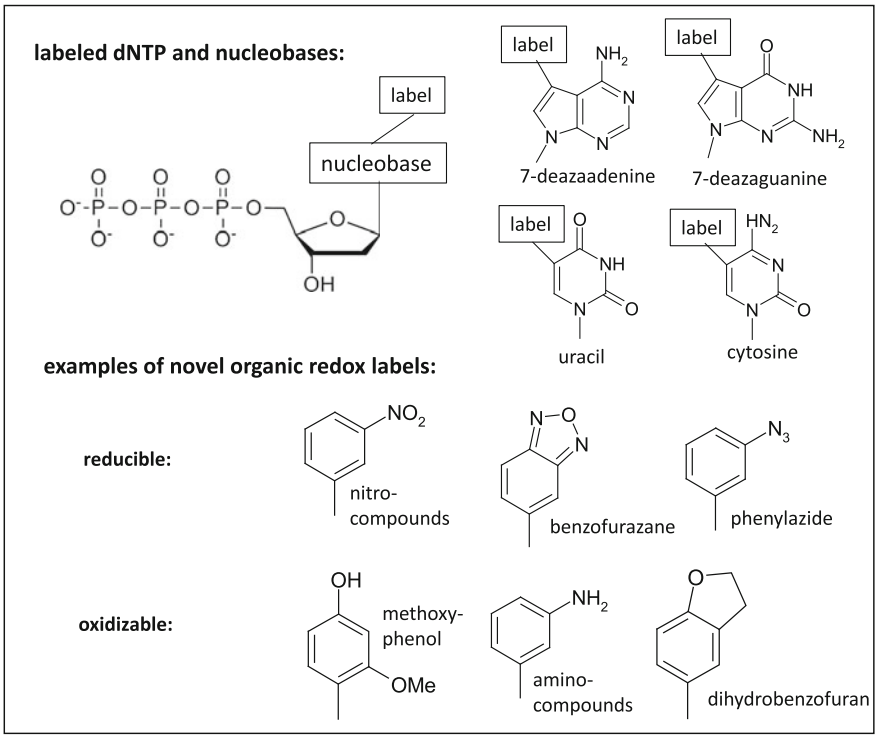

Fig. 2 Scheme of base-modified deoxynucleoside triphosphates and examples of nitrogenous and/or oxygenous organic compounds designed as electrochemically reducible or oxidizable labels

3.2.2 Construction of labeled DNA using polymerases and modified dNTPs

Primer extension. DNA-dependent DNA polymerases, key enzymes involved in DNA replication as well as repair processes, work on the principle of template sequence-driven attachment of a nucleotide (using dNTP as an activated precursor) to terminal 3‘–OH group of RNA or DNA primers (Fig. 3a). Using this primer extension (PEX) reaction and suitable modified dNTPs, labeled DNA can easily by prepared with the modified nucleotides incorporated sequence-specifically according to the template (complementary) sequence [57, 98]. It was shown that Klenow fragment of bacterial DNA polymerase I and a number of thermostable DNA polymerases belonging to the “B-family” (e.g., Vent, Pwo, KOD) tolerate the presence of a number of modifications on sugar as well as at 4-position of pyrimidines or 7-position of 7-deazapurines. Some limitations in the PEX efficiency may arise from steric effects when two or more base-modified nucleotides bearing bulky external substituents are incorporated in adjacent positions [35]. Nevertheless, for a preparation of multiply tail-modified probes (in analogy to the

above mentioned probes chemically modified with Os(VIII),L [63]) it is easy to design suitable templates (e.g., to avoid homonucleotide clusters) and optimize the modification process.

Polymerase chain reaction and two-step DNA modification. The polymerase chain reaction (PCR) is widely used for amplification of specific DNA fragments for preparative or diagnostic purposes. The technique has successfully been utilized for the preparation of DNA fragments bearing e.g., 7-deazapurines as analogues of natural purine bases [109], biotinylated nucleotides [60] or some relatively simple base-modified nucleotide conjugates such as those bearing formylthiophene moieties [100, 101]. However, in other cases the preparation of densely modified PCR products is even more challenging than in PEX, because, in addition to the above mentioned problem with clustered incorporation of bulky adduct into the daughter strand, in PCR the polymerase must cope with reading of the modified template strand. When rather bulky modifications are to be incorporated into PCR products, these obstacles can be circumvented by a two-step DNA modification protocol: in the first step, relatively small reactive groups are introduced by PCR, which are subsequently used for DNA postsynthetic modification with the ultimate labels. This approach was successfully applied for the preparation of hydrazone-modified DNA, where the reactive moiety incorporated by the enzyme was formylthiophene and the formyl groups were then reacted with hydrazine derivatives [100, 101].

Terminal transferase catalyzed DNA tailing. Terminal deoxynucleotidyl transferase (TdT) is a template-independent DNA polymerase synthesizing ss tails via consecutive attachment of nucleotides (using dNTPs as substrates) at the 3‘-OH end of ss or ds primers (initiators, Fig. 3b). TdT has been used in the area of recombinant DNA construction to add sticky ends to cloned DNA fragments [110]. It has been demonstrated that the enzyme can also be used for synthesis of modified DNA tails to introduce multiple redox tags into hybridization probes or substrates for protein-DNA interaction studies [111].

3.2.3 Utilization of specific features of the redox DNA labels

Multipotential redox coding of nucleobases and SNP typing. Since reduction of natural nucleobases takes place at relatively highly negative potentials (on mercury/amalgam electrodes) and oxidation at rather positive potentials (on carbon electrodes), there is a relatively wide region of potentials available for measuring electrochemical signals of various DNA labels without interference with the intrinsic DNA signals (Fig. 4). The labels can be chosen according to potentials of their reduction and/or oxidation so that their signals do not mutually overlap and the signals can be measured independently on each other. Hence, a combination of different redox labels can be utilized in electrochemical typing of SNPs, as demonstrated previously for a combination of nitrophenyl, $[Os(bipy)_3]^{3+/2+}$ and aminophenyl to encode three different nucleobases [35] or, quite recently, for a combination of ferrocene, anthraquinone, methylene blue and phenothiazine to complete the set of all four DNA bases [103]. Besides differences between the

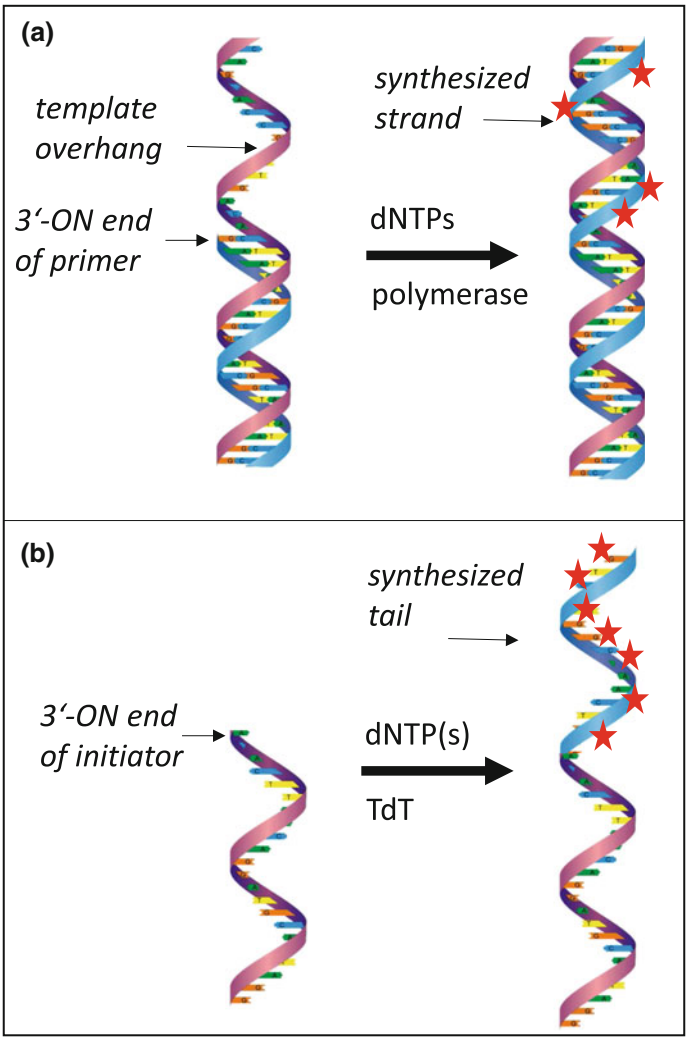

Fig. 3 Scheme of enzymatic incorporation of labeled nucleosides into DNA: **a** primer extension; **b** terminal deoxynucleotidyl transferase (TdT) tailing

redox potentials, also the reversibility or irreversibility of corresponding electrode process can be used for unequivocal identification of the given redox-encoded nucleobase. Combination of two or more independently detectable labels can also be utilized for ratiometric analytical techniques, suitable for the determination of relative changes of the abundance of different nucleobases in a DNA sequence stretch [70]. This approach to the detection of mutations, in which one redox-coded nucleobase is used as an internal standard to determine alterations in the number of another one, overcomes a general problem of microanalytical techniques with

difficult control of the total analyte (DNA) concentration (unlike the absolute intensity of a signal, the ratios of signal of two labels introduced in the same DNA molecule are concentration-independent).

Switchable redox labels. Specificity of redox DNA labeling can be further increased by using moieties which are either electrochemically, or chemically convertible to products which exhibit electrochemical activity different from that displayed by the originally introduced species. For example, product of the irreversible four-electron reduction of nitrogroup is a corresponding hydroxylamine derivative, which can be oxidized to nitroso derivative in a reversible two electron-two proton process [104, 112]. Such signal switching has been demonstrated to solve a problem with overlapping reduction signals of nitrophenyl and anthraquinone, which preclude their distinction during the primary reduction but can be determined independently after conversion of the nitro group into the hydroxylamino/nitroso system (see Fig. 4 for relative peak positions). Signals due to the latter redox processes can be switched on only via reduction of the nitro derivative and thus do not interfere before applying sufficiently negative potentials [104]. Similarly, the guanine signal measured on mercury electrodes is "activated" only upon applying potentials more negative than −1.6 V and thus does not appear and interfere with other analytical signals until it is desirable.

Another way to switching electrochemical signals on/off involves chemical conversion of polymerase-introduced moieties into the ultimate labels. Reaction of aldehyde groups with hyrazine to form hydrazones (Fig. 5a), mentioned above in

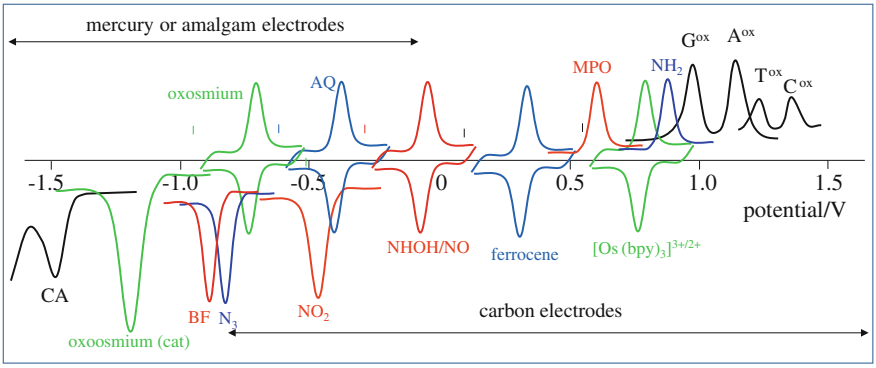

Fig. 4 Schematic representation of reduction and oxidation signals of unmodified DNA (*black curves*; CA, peak due to reduction of A and C residues; G^{ox}, A^{ox}, T^{ox} and C^{ox}, signals due to oxidation of corresponding nucleobases) and electrochemical responses of selected DNA labels (in *color*). The signals are shown on potential scale (potential values measured against Ag/AgCl/3 M KCl reference electrode). *AQ* anthraquinone, *MPO* methoxyphenol, *BF* benzofurazane, N_3 azidophenyl, NO_2 nitrogroups; NH_2 amino groups, *NHOH/NO* hydroxylamino/nitroso redox couple generated via reduction of nitrogroup; oxoosmium—NA adducts with Os(VIII),L or Os (VI),L reagents; oxoosmium (cat)—osmium reduction coupled to catalytic hydrogen evolution. Irreversible oxidations and irreversible reductions are denoted by positive or negative peaks, respectively, reversible redox processes by peak-counterpeak pairs

relation to two-step DNA labeling techniques, is one of typical examples: due to such conversion, the formyl-specific reduction signal decreases while signals due to electroactive moieties present in the reaction products appear (i.e., the hydrazone C = N bond and groups attached via the hydrazone linkage—nitro groups or and/or benzofurazane, as presented in [100]). From relative changes of the primary and secondary signals, the conversion degree can be deduced. Another example comprises polymerase-incorporated phenylazide group (yielding a strong reduction signal on mercury-based electrodes) click-transformable into corresponding substituted triazoles in reactions with acetylene derivatives (Fig. 5b) [105]. The triazole group has been found to be electrochemically "silent", hence after the click reaction, the modification-specific (phenylazide) signal disappears. When an acetylene derivative bearing another redox-active moiety (such as nitrophenyl) is used for the click reaction, the resulting product yields a signal specific for the latter; thus, similarly as above, the conversion degree can be assessed from relative changes of the disappearing (phenylazide) and appearing (nitrophenyl) signals. This system has been utilized to monitor shielding effects of a DNA-binding protein [105].

4 DNA Labeling with Enzymes and Biocatalytical Signal Amplification

Enzymes as biocatalytic labels were introduced in connection with various immunoassays, employing various enzyme-antibody conjugates, usually in sandwich detection systems (e.g., enzyme-linked immunosorbent assays commonly well-known as ELISA, or western immunoblot assay). Enzymes applied in these techniques include, for example, horse radish peroxidase, β-galactosidase or alkaline phosphatase, and substrates chosen according to the detection technique applied, with colorimetry or enhanced chemiluminiscence featuring typical examples. The enzyme-linked techniques can also be coupled to electrochemical detection of product of the enzyme reactions [113, 114]. Since one molecule of the enzyme can catalyze conversion of many molecules of (electrochemically silent) substrate into many molecules of (electroactive) indicator, the enzyme-linked detection systems are inherently offering a high sensitivity of the target molecule detection. Typical examples of these indicators are electrochemically oxidizable phenols (e.g., 1-naphthol) or reversible redox p-aminophenol, both released by alkaline phosphatase from corresponding inactive phosphoesters. To attach the enzymes to NA, bioaffinity (biotin-streptavidin or antibody-hapten) interactions are usually utilized.

a. DNA modification with biotin for enzyme-linked electrochemical assays
DNA hybridization assays employing biotin-labeled signaling probes with subsequently attached streptavidin-alkaline phosphatase conjugate (SALP) have been successfully used in various experimental modes. One group of techniques have been based on application of magnetic beads, on which the capture probe-biotinylated target DNA-SALP conjugate was assembled and the

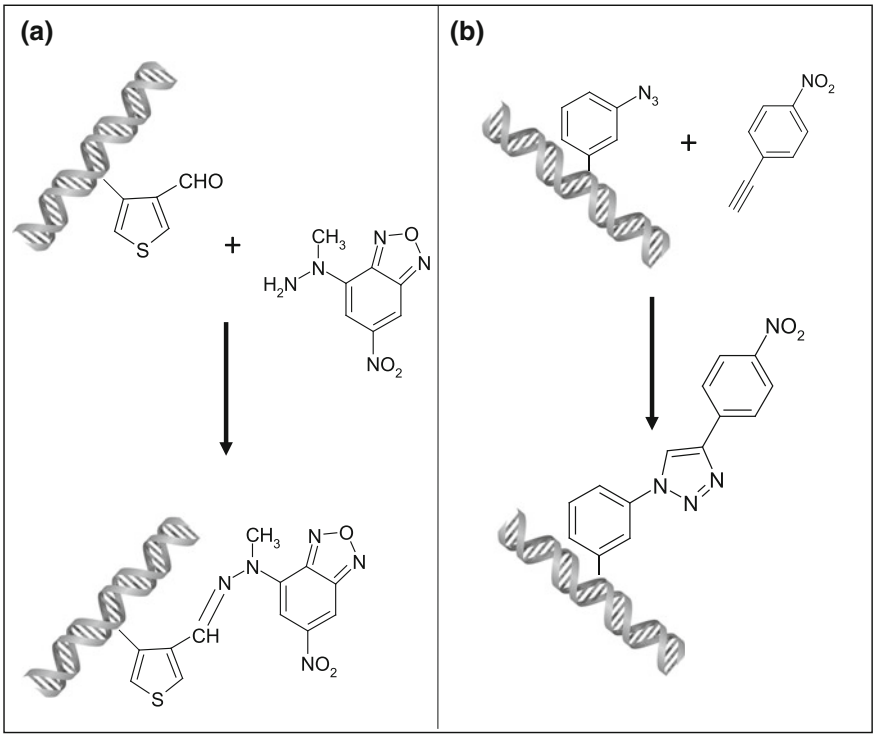

Fig. 5 Postsynthetic modification of DNA functionalized with reactive groups: **a** hydrazone formation between formylthiophene enzymatically incorporated into DNA (see Fig. 3a) and a hydrazine derivative bearing nitrobenzofurazane as a redox label; **b** click reaction to convert azidophenyl moiety on DNA into triazole bearing electrochemically active nitrophenyl group

electroactive indicator generated, being subsequently determined voltammetrically in the supernatant [18, 34]. Other techniques have involved adsorption of target DNA at a carbon (pyrolytic graphite or screen-printed) electrode surface, hybridization with biotinylated probe, attachment of SALP and enzymatic generation of the soluble indicator directly at the electrode, with subsequent voltammetric measurement directly in the substrate solution. The latter approach appeared useful for a fast, simple and relatively inexpensive analysis of real PCR-amplified DNA samples, including amplified cDNA resulting from reverse transcription of RNA to monitor gene expression [28]. Besides utilization of commercially available biotinylated ON probes, the biotin affinity tags can be incorporated into DNA using any of polymerase-based techniques mentioned in Sect. 3.2. and biotin-labeled dNTPs. Hence, it has been possible to apply analogous techniques to detect SNP in real PCR-amplified cDNA via sequence-specific PEX incorporation of biotinylated uridine or cytidine [34], or to monitor PCR reactions via incorporation of biotinylated nucleotides [60]. Biotin-based techniques have been proposed also for the detection of DNA damage e.g., in a variant of the TUNEL test [47].

b. Electrochemical immunoassays of haptene-modified DNA
Digoxigenin (DIG) is a haptene commonly used for DNA labeling in immunoassays, including in situ hybridization. Similarly as biotin, it can be introduced into DNA either during commercial chemical synthesis of ONs, or incorporated by polymerases using a DIG-labeled dNTP (see Sect. 3.2). It is detected using anti-DIG-antibodies, which are commercially available in the form of enzyme (e.g., ALP) conjugates. As a recent example, a dual bioaffinity detection system was proposed to detect DNA ligation [115], in which a DNA half-strand is attached via biotin to streptavidine-coated magnetic beads, and the ligation reaction is detected by DIG attached to opposite end of the other half strand. In principle analogous techniques can be applied to detect breakage of the DNA molecules.

A specific type of haptene, for the detection of which antibodies are available [116], is the Os(VIII),bipy-thymine adduct. The antibody recognizing osmium-modified DNA was utilized previously in an electrochemical enzyme-linked DNA hybridization assay [65]. Os(VIII),bipy-pretreated target DNA was hybridized at the MB via a homopurinic stretch and the osmium tags were detected by the enzyme-linked immunoassay using monoclonal antibody OsBP7H8 and a secondary antibody-ALP conjugate. Such system encompasses two levels of amplification (multiple osmium adducts to accommodate multiple enzyme molecules, and the enzyme biocatalysis). This immunodetection system is in principle applicable with any Os(VIII),bipy based technique mentioned in Sect. 3.1.1, including DNA damage assays (for example, it was successfully applied to detect thymine residues in the apurinic sites) [90].

5 Conclusions

Electrochemical analysis offers a variety of ways to analyze nucleotide sequences via hybridization and/or polymerase-based techniques, as well as to analyze DNA damage and DNA interactions with genotoxic agents. These applications may employ either label-free electrochemical NA sensing based on intrinsic electrochemical properties of natural NA components, or may be based on applications of various electrochemically active labels or indicators. Although the label-free techniques have found a variety of applications in detection of both DNA hybridization and DNA damage, the redox or enzyme labels exhibit a great potential to improve analytical parameters of the NA electrochemical analysis. In particular, labeling of specific nucleotide sequences, or even individual nucleobases, with diverse redox tags (in fact featuring DNA or nucleobase coding by redox potentials of the labels, which differ from redox potentials of natural NAs and from each other), greatly increase selectivity of electrochemical detection of target sequences and/or single base polymorphisms. Structure selectivity of NA modification with redox-active chemical probes such as oxoosmium complexes has been shown to increase considerably sensitivity of electrochemical methods used to detect DNA lesions such as abasic sites or single base mismatches. Recent progress

in organic synthesis of labeled nucleoside triphosphates has extended the possibilities of polymerase-based DNA labeling and offered novel types of electroactive DNA tags, including electrochemically or chemically switchable ones. Last but not least, labeling of NA with enzymes generating electroactive indicators, in connection with techniques of NA modification with bioaffinity tags to attach the enzymes, has been utilized to develop highly sensitive enzyme-linked NA assays.

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