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Electrochemical nucleic acid-based biosensors: Concepts, terms, and methodology (IUPAC Technical Report)*

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Abstract: An electrochemical nucleic acid (NA)-based biosensor is a biosensor that integrates a nucleic acid as the biological recognition element and an electrode as the electrochemical signal transducer. The present report provides concepts, terms, and methodology related to biorecognition elements, detection principles, type of interactions to be addressed, and construction and performance of electrochemical NA biosensors, including their critical evaluation, which should be valuable for a wide audience, from academic, biomedical, environmental, and food-testing, drug-developing, etc. laboratories to sensor producers.

Keywords: aptamers; biosensors; electrochemistry; DNA damage; IUPAC Analytical Chemistry Division; nucleic acids; nucleic acid hybridization; nucleic acid interactions.

CONTENTS

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1. INTRODUCTION

The previously published IUPAC technical report "Electrochemical biosensors: Recommended definitions and classification" [1] did not deal extensively with nucleic acids (NAs) [2] as the biological recognition element, but considered the work on other types of biosensors. Since that time, significant progress in the development and application of electrochemical sensors based on deoxyribonucleic acid

(DNA) and other NAs, including aptamers and peptide NAs, has been achieved. On the other hand, so far there have been no efforts at essential classification in this dynamically developing field.

An **electrochemical NA-based biosensor** is a device that integrates an NA (natural and biomimetic forms of oligo- and polynucleotides) as the biological recognition element and an electrode as the physicochemical transducer. In this regard, the previous IUPAC report on electrochemical biosensors [1] is fully acceptable for electrochemical NA-based biosensors, including the biosensor definition and information obtained. NA-based biosensors belong to the family of **chemical sensors**, which transform (bio)chemical *stimulus* from an analyte in relation to NA into the analytically useful information (analyte type/concentration, NA chemical structure and/or its change, etc.). From the point of view of electroanalytical chemistry, the electrochemical NA-based biosensor represents an NA-modified electrode which is used mostly in voltammetric and chronopotentiometric detection modes [3]. Electrochemical impedance spectroscopy (EIS) is also used as the investigation and detection technique [4]. The use of the concept of an electrode modified with the DNA layer has allowed a significant decrease of the amount of DNA tested/determined.

Comment: Use of terms "**sensor**" and "**sensing**" (or assay) is sometimes confusing. Here, it is necessary to distinguish strictly between NA biosensors and NA sensing. While in an **electrochemical NA biosensor**, the NA has to be in an intimate contact with the electrode prior to and during the NA interaction with an analyte, the **NA electrochemical sensing** has broader meaning. Product of an interaction of any NA with an analyte (generated either in solution or at another surface) or NA itself can be detected or its concentration determined electrochemically, usually after accumulation onto the electrode surface. Most of the electrochemical NA biosensors mentioned in this report employ detection principles which can be applied in alternative electrochemical biosensing (micro) techniques as well. In some cases, the latter techniques can be similar to or even more efficient than the biosensors. For example, DNA damage or association interactions can easily be monitored using simple ex situ (adsorptive transfer stripping) electrochemical analysis of DNA exposed to a damaging agent or interacting in solution (prior to adsorption at an electrode). A specific class of approaches that have complemented the classical concept of electrochemical DNA sensors during the last decade employs magnetic beads as the surface on which the DNA biorecognition event (hybridization, interaction with protein, etc.) occurs. Then, target DNA, signaling probe, or other indicator molecules captured or generated at the surface of the beads can be determined electrochemically. Such techniques are referred to as "double-surface" ones due to the two different surfaces involved (one—the magnetic beads—for the biomolecular interaction, and the other—the electrode—for detection) [5]. A more detailed description of the double-surface strategy is beyond the scope of this report.

According to [1], biosensors can be classified considering the biological specificity—conferring mechanism, mode of signal transduction, and analytes or reactions that they monitor. The classification of the biosensors according to a third point of view (i.e., analytes or reactions) is also acceptable for the electrochemical NA-based biosensors. However, the ratio of the utilization of the NA biosensors is shifted from the detection of analytes more to the reactions of NA when compared to the enzyme- and immuno-sensors. In other words, the NA biosensors discussed in this report often deal with the investigation of characteristic NA interactions rather than with the conventional determination of the concentration of an analyte (a measurand).

Specificity of the biosensor response can also be accepted as a typical feature of the device, taking advantage of the DNA strands bioaffinity properties. Typically, specificity/selectivity is induced by both NA surface film and chemical properties of an analyte. The NA-based biosensors are specific either to the analyte (nucleotide bases sequence, protein) or to the NA itself (its damage).

2. BIORECOGNITION ELEMENTS

2.1 Nucleic acids used at biosensors

Today, numerous types of natural and synthetic DNA and RNA molecules are available for electrochemical biosensors, including chromosomal DNA as well as well-defined viral or plasmid NAs. The plasmid and the viral DNA molecule can be cleaved into the fragments of various lengths by the action of restriction endonucleases and/or amplified by polymerase chain reaction (PCR). End-labeled DNAs, polynucleotides with random or monotone sequences, and synthetically prepared oligonucleotides with programmable sequences (allowing modification of bases and/or backbones) are also commercially available. Oligonucleotides with RNA backbone are currently more expensive than oligodeoxyribonucleotides (ODNs).

Note: At present, mainly synthetic ODNs are used as **probes** in the DNA hybridization sensors. End-labels, such as thiols, disulfides, amines, or biotin, are incorporated to immobilize ODN to transducer surfaces. A long flexible spacer is usually added to provide sufficient accessibility for surface attachment. Hydrocarbon linkers are frequently used for this purpose. Selection of the probe nucleotide sequence depends very much on the target sequence. Certain specific applications require the right choice of probe length. For example, in discrimination of single-base mismatches, shorter probes are preferred because a single-base mismatch is more likely to disturb the stability of a short DNA duplex and eventually prevent its formation.

Peptide nucleic acid (PNA) is a synthetic DNA mimic, which contains 2-aminoethylglycine linkages instead of the negatively charged phosphodiesteric backbone of ODNs. The PNA probes are particularly convenient for the detection of single-base mismatches (point mutations, SNPs) because the stability of DNA-PNA duplexes is strongly influenced by a single-base mismatch [6]. Other kinds of synthetic NA, such as locked nucleic acid (LNA), are also used.

Comment: In real DNA analyses, PCR-amplified genomic DNA segments are mostly used as target DNAs. On the other hand, in a large number of papers, synthetic ODNs serve as target DNAs. This may be acceptable if in principle new technology is being developed. Otherwise, natural amplified or nonamplified target DNAs should be used to validate the new detection principles in analysis of real biological material. Target DNAs can be labeled, which in the case of natural DNAs can be more difficult than in the case of synthetic ODNs. Osmium tetraoxide complexes [5,7] are particularly suitable for labeling of both natural and synthetic DNAs, RNAs, as well as for PNA end-labeling. Sometimes, it may be convenient to work with unlabeled target DNA. In such a case, either label-free detection is applied or labeled signaling (reporter) probes are used. Occasionally, the DNA probe may serve only as a capture probe (CP) and the signaling probe detects presence of the specific nucleotide sequence. Signaling probes are usually synthetic ODNs.

2.2 Nucleic acid aptamers

Nucleic acid aptamers are single-stranded (ss) oligonucleotides (mainly DNA or RNA) originating from in vitro selection, which, starting from random sequence libraries, optimize the NAs for high affinity binding to a given target [8–10]. The term "aptamer" derives from the Latin *aptus*, "to fit", and emphasizes the relationship between aptamer and its target. Aptamers, upon association with their target, fold into complex three-dimensional shapes in which the target becomes an intrinsic part of the NA structure.

Comments: The term "aptamer" should be clearly distinguished from other terms such as "ribozyme", "DNAzyme", and "aptazyme", which are defined as follows. Ribozymes are catalytic RNAs; some ribozymes have been found in Nature and mediate phosphodiester bond cleavage and peptide bond formation. In vitro selection has been used to generate RNA enzymes with novel structures and catalytic functions (i.e., Diels–Alder reactions, biphenyl isomerization, C–S bond by Michael reaction, etc.). DNAzymes are DNA-based catalysts that have not been found in Nature and are generated only by in vitro selection. The ligand-binding and catalytic features of NA can be combined to generate allosteric ribozymes or "aptazymes". When ligands bind to an aptazyme, conformational changes in the ligand-binding domain are transduced to a change in the catalytic core and a concomitant modulation of catalytic activity. The term "aptamer" has been recently used also to denominate a new class of peptidic bioreceptors. To avoid misunderstanding in this report with the term, aptamers are only considered NA-based aptamers.

Note: In vitro selection is an iterative method mainly known as Systematic Evolution of Ligands by EXponential enrichment (SELEX) developed by the Gold, Ellington, and Szostak laboratories in the early 1990s [11,12].

The properties of aptamers make them an attractive class of molecules that meet and exceed the properties of antibodies for biosensor development. Both DNA and RNA aptamers bind their targets with dissociation constants (K_d) which in the case of proteins are in the low picomolar to low nanomolar range, discriminating between related proteins that share common sets of structural domains. Affinities in the micromolar range can be observed in the case of aptamers—small molecule complexes. The characterization of affinity of aptamer binding is required for estimating the sensitivity and selectivity of the appropriate biosensors, also called "aptasensors".

Aptamers with affinity for a large variety of molecules, including virtually any class of proteins (enzymes, membrane proteins, viral proteins, etc.), peptides, drugs, toxins, low-molar-mass ligands, and ions have been isolated. The folding of NA around the target provides numerous discriminatory intermolecular interactions. These interactions fall in the class of noncovalent bonding and are mainly stacking, shape complementarity, electrostatic, and hydrogen-bonding interactions. Multiple interactions contribute to the same aptamer-target complexes. The molecular interactions govern the specific recognition of and discrimination between different target classes in aptamer complexes [8–10]. The following advantages of aptamers are mostly counted over other biorecognition elements: (i) chemical synthesis, which does not require biological raw materials (bioethics requirements); (ii) universal approach to selection procedure, which does not depend on a particular analyte (possibility to use toxins as well as molecules that do not elicit a good immune response); (iii) cost-effective production; (iv) high affinity and molecular discrimination; (v) high thermal stability and opportunities for the further modification that provides the immobilization of aptamers onto solid support and mild alteration of specificity and selectivity of the binding.

3. DETECTION PRINCIPLES

3.1 Electroactivity of nucleic acids

3.1.1 Reduction and oxidation of nucleic acids

The electrochemical activity of NAs (both the native high-molecular ones as well as oligonucleotides with rather short sequences) is conferred by the electroactivity of its components, nucleobases and sugar residues [3]. At **mercury-based electrodes**, adenine (A) and cytosine (C) residues in ssNAs undergo reduction processes close to –1.4 V (against SCE) in neutral or weakly acidic medium (giving rise to peak CA, Fig. 1A). In cyclic voltammetric modes, chemically reversible reduction of guanine (G) in

DNA and RNA leads to an anodic peak G (at about –0.3 V, Fig. 1A). Nucleobase residues in DNA and RNA are reduced in an adsorbed state. All NA bases have been reported to be electrochemically oxidized at **carbon electrodes**, but only adenine and (particularly) guanine oxidation peaks (Fig. 1A) have been widely utilized in NA biosensors. The oxidation of sugar residues in NA at copper electrodes has also been employed.

Comment: The electroactivity of NAs on the mercury electrodes has not been fully utilized because of concerns regarding the toxicity of metal mercury. Recently, it has been shown that with nontoxic solid dental amalgam electrodes, electrochemical responses similar to the hanging mercury electrode can be obtained [13,14]. There were also few reports about the guanine residue oxidation at gold electrodes [15].

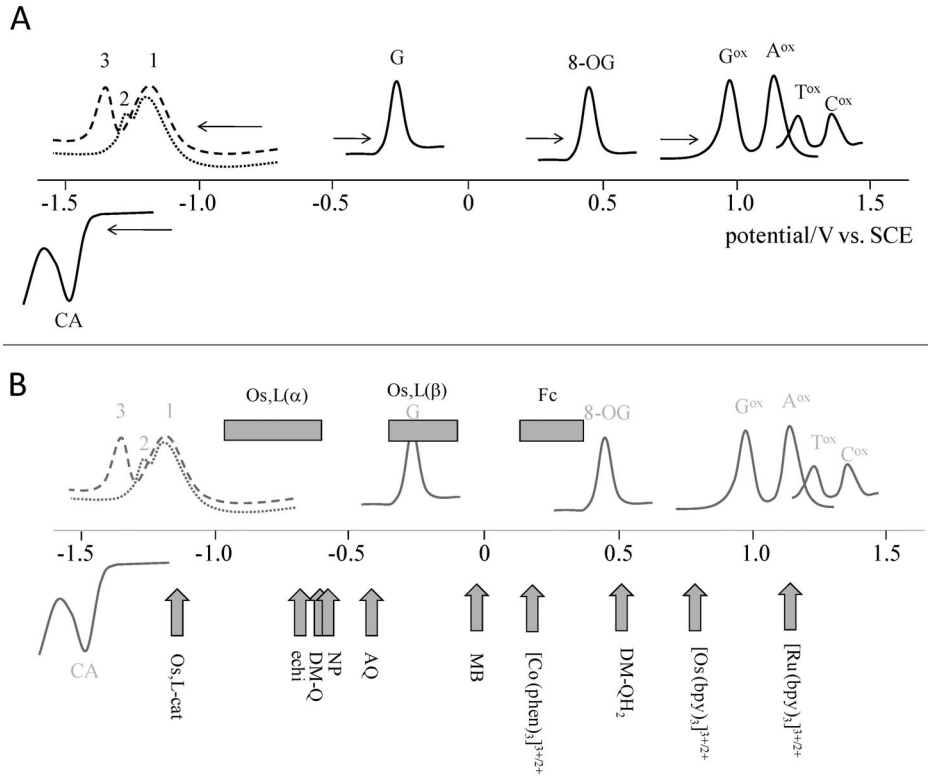

Fig. 1 (**A**) Scheme of intrinsic electrochemical responses of DNA. Solid curves represent voltammetric peaks due to reduction or oxidation of nucleobase residues in the DNA at mercury/amalgam electrodes (CA: reduction of cytosine and adenine, G: re-oxidation of an electrochemically generated reduction product of guanine) or carbon electrodes (G^{ox}, A^{ox}, T^{ox}, C^{ox}: oxidation of standard nucleobases guanine, adenine, thymine, and cytosine; 8-OG, oxidation of a common product of oxidative DNA damage, 8-oxoguanine). Dotted or dashed curves show capacitive (tensammetric) voltammetric responses of dsDNA lacking or containing free ends, respectively, at mercury-based electrodes (peak 1 is due to adsorption/desorption processes of the DNA sugar-phosphate backbone, while peak 3 is due to single-stranded DNA segments adsorbed via bases). (**B**) Scheme of redox potentials of some electroactive species used as DNA labels/indicators (shown relative to the intrinsic DNA responses): Os,L-cat, catalytic signal of DNA labeled with osmium tetraoxide complexes (Os,L); echi, echinomycin; DM-Q, quinone moiety of daunomycin; NP, nitrophenyl; AQ, anthraquinone; MB, methylene blue; DM-QH_2, hydroquinone moiety of daunomycin. Bars in the upper part of the scheme show potential regions in which redox electrochemistry is yielded by "tunable" DNA labels such as Os,L (their redox potentials depend on the ligand L) or ferrocene (Fc; its redox potential can be tuned by different substituents and/or electronic conjugation with another aromatic system).

3.1.2 Tensammetric responses of nucleic acids

The polyanionic nature of the NAs causes them to undergo characteristic adsorption/desorption (reorientation) processes at the mercury-based electrodes upon applying (changing) negative potentials due to interplay between electrostatic repulsion and relatively strong adsorption via hydrophobic parts of the polynucleotide chains (particularly bases) [3,16]. In weakly alkaline media, these processes give rise to analytically useful tensammetric (capacitive) [2] current signals (Fig. 1A) that sensitively reflect changes in the NA structure. DNA sugar-phosphate backbone undergoes adsorption/desorption processes around –1.2 V (vs. SCE) yielding peak 1. Peak 2 (at –1.3 V) has been ascribed to distorted DNA double-helical segments with partially accessible edges of base pairs, which thus take part in the adsorption/desorption processes. ssDNA segments with freely accessible bases produce peak 3 close to –1.45 V (Fig. 1A).

3.2 Effects of DNA structure

Marked differences in the **voltammetric** and **tensametric responses** of native (double-stranded, ds) and denatured (ss) DNA have been observed at the mercury electrodes under certain conditions [3,16]. Large current responses of ssDNA as compared to very small ones of dsDNA are explained by inaccessibility of the nucleobases in dsDNA for adsorption and the electroactive sites of cytosine and adenine for the reduction at the mercury electrode. Both faradaic and tensammetric [2] responses measured at the mercury-based electrodes thus sensitively reflect subtle changes in DNA structure, resulting in uncovering the nucleobases. On the other hand, the primary oxidation sites of guanine and adenine are relatively well accessible in the dsDNA, making the oxidation responses at carbon electrodes less sensitive to DNA structure changes.

3.2.1 Changes of DNA structure at charged electrode surface

Using the faradaic and capacitive DNA responses, it was shown that at neutral and weakly alkaline pH values prolonged contact of dsDNA with the surface of the mercury electrode within a narrow potential region around –1.2 V vs. SCE resulted in a relatively slow, irreversible opening of the DNA double helix at the surface [3,16]. No extensive duplex opening was found in covalently closed circular DNAs in agreement with an assumption that DNA unwinding starts from DNA strand ends.

Note: DNA unwinding was also observed at negatively charged gold and silver electrodes and at other surfaces.

3.3 Label-free techniques utilizing electrochemical and/or surface activity of nucleic acids

Nucleic acids are electrochemically active due to the presence of electrochemically oxidizable or reducible nucleobases, and they exhibit specific surface activity depending on which NA components take part in adsorption at the electrode surface. Electrochemical analysis of the DNA can thus in principle be conducted without introducing specific labels.

3.3.1 Guanine oxidation at carbon electrodes

Although all common nucleobases have been reported to be electrochemically oxidizable at carbon electrodes and adenine, cytosine, and guanine to give electrochemical responses at mercury-based electrodes, the vast majority of label-free DNA biosensors employ oxidation of the guanine moiety at carbon or other solid electrodes as the source of analytical signals [3]. This choice is dictated by (a) relatively low redox potential of guanine, making it easily detectable by direct electrochemical oxidation without using additional reagents; (b) the fact that guanine is the most frequent target for many DNA damaging species and its chemical modification is often accompanied by a loss of the guanine peak.

Comment: Limitations of the guanine redox process-based label-free and reagent-less sensors arise from (a) usually insufficient discrimination between the probe and target strands in DNA hybridization assays due to almost even distribution of all four nucleobases in most natural nucleotide sequences (see Section 4.1); (b) relatively low sensitivity toward small local changes in DNA structure such as strand breakage (see Section 3.2); and (c) the fact that only guanines in the close vicinity of the electrode surface can undergo direct electrooxidation. The latter obstacle can be overcome by applying soluble **redox mediators** such as rhodium or ruthenium complexes that can shuttle electrons from guanine residues in distant parts of DNA chains to the electrode [17,18]

$$G_{(DNA)} + M^{z+} \rightarrow G^{\bullet+}{}_{(DNA)} + M^{(z-1)+}$$

$$M^{(z-1)+} - e^- \rightarrow M^{z+} \text{ (electrochemically)}$$

3.3.2 Structure-sensitive responses of DNA at mercury-based electrodes

Reduction and tensammetric responses of NAs at the mercury and some amalgam electrodes are strongly dependent on the hydrophobic nature of bases, on whether they are present in ss or ds regions of the NAs, and/or whether the DNA adsorbed at the electrode surface can undergo a structural transition connected with a change in the nucleobase accessibility [3,16]. Based on these principles, denaturational transitions, strand breaks (making the double helix susceptible to potential-induced surface denaturation) or ss regions within dsDNA can be detected by AC voltammetry or impedance techniques with a considerable sensitivity (such as one strand break per 2×10^5 nucleotides in circular duplex DNA).

Note: Thus, mercury-based electrodes modified with circular duplex DNA have been designed as label-free and reagent-less sensors for DNA damage involving (or convertible to) strand breaks (see Section 4.3.1).

3.4 Noncovalent redox indicators

Despite the analytical usefulness of the intrinsic NA electrochemical activity, a number of detection techniques have employed electroactive species serving as redox indicators of the events (such as hybridization, damage, complex formation with another substance) having undergone by the DNA at the electrode surface.

Comment: Development of these detection techniques (which are still label-free in the sense of no chemical modification of NA probes or targets) has been motivated by several limitations of the intrinsic DNA electroactivity-based approaches (see Section 3.3):

(a) only mercury or some amalgam electrodes possess a sufficiently negative potential window to observe reduction and tensammetric responses of unlabeled NA, and only carbon electrodes can be used for direct electrochemical detection of the nucleobase oxidation. Other electrodes, including very popular gold ones, exhibit insufficient hydrogen overvoltage on the negative side and electrooxidation of the electrode material at insufficiently positive potentials to allow detection of well-defined NA responses. On the other hand, typical redox indicators are reduced/oxidized at less extreme potentials (Fig. 1B), thus extending the choice of working electrodes.

(b) electrochemical reduction and oxidation of NA bases is irreversible and thus preventing reusability of the sensors (recognition layers). This obstacle can be overcome by using redox indicators, producing their (often reversible) electrochemical responses at "safe" potentials.

Electrochemically (redox) active substances binding preferentially to either ssDNA or dsDNA have been applied as indicators of DNA hybridization (to recognize an ss probe from the hybrid duplex) or DNA damage (to recognize intact dsDNA from degraded DNA that has lost its double-helical structure) [3,16,17]. The noncovalent redox indicators encompass species interacting with DNA electrostatically, DNA intercalators, or groove binders (for more details on DNA association interactions, see Section 4.2). Some of the noncovalent indicators can act as redox mediators (electron shuttles) and/or electrocatalysts, which may be either soluble and diffusionally free (see Section 3.1), or connected to the dsDNA base pair stacks and take part in electron transfer mediated by the DNA double helix.

The **electrostatic indicators** respond to differences in negative charge density between ssDNA and dsDNA. Hybridization between a surface-confined probe with target DNA results in increase of the negative charge density, while degradation of dsDNA covering the surface results in the opposite effect. Using peptide nucleic probe (bearing no negative charge) offers a better discrimination between the (uncharged) probe and the (negatively charged PNA·DNA) hybrid. Redox indicators such as the anionic complex hexacyanoferrate (III/II), $[Fe(CN)_6]^{3-/4-}$, (being repelled from the DNA-modified surface) or the cationic complex $[Ru(NH_3)_6]^{3+/2+}$ (being attracted to the negatively charged hybrids) have been employed to monitor changes in the negative charge density using impedimetric or voltammetric techniques as well as scanning electrochemical microscopy [17,19,20].

Note: Some authors use the term "redox probe" for the $[Fe(CN)_6]^{3-/4-}$ indication system. It is recommended to avoid using the term "probe" in the context of DNA sensors due to possible confusion with the "hybridization probe".

Groove binders [such as bis-benzimide (fluorescent dye Hoechst 33258)] and **intercalators** (such as daunomycin, metal chelates with condensed aromatic nitrogenous heterocyclic ligands, phenoxazines, etc.) recognize specific structural features of dsDNA and bind with a higher affinity to the duplex NA, which results in selective accumulation of the indicators in dsDNA (after hybridization or prior to damage to the duplex DNA) layer at the electrode surface, thus increasing the peak currents of the indicators. To improve discrimination between ssDNA and dsDNA, bis-intercalators (e.g., echinomycin) or threading intercalators [e.g., *N*,*N*-bis[3-(3-ferroceneacetylaminopropyl)methylaminopropyl]naphthalene-1,4,5,8-tetracarboxylic acid diimide (ferrocenyl naphthalene diimide)] have been applied, which form thermodynamically or kinetically more stable complexes with dsDNA than the simple intercalators [16,17,21].

Substances associating preferentially with ssDNA have also been applied in electrochemical sensors for the DNA hybridization. A typical example is a phenothiazine dye methylene blue (MB) which has been reported to associate with unpaired guanine residues. In dsDNA this interaction is hampered, which results in decrease of the current due to MB reduction [22].

3.5 Covalently bound labels

Introducing an electrochemically (directly or indirectly) detectable label (tracer) into the NA considerably improves specificity of the assay (typically a hybridization one) because the labeled NA can easily be distinguished from the unlabeled one (e.g., labeled target DNA from unlabeled CP, labeled signaling probe from unlabeled target, or a labeled nucleotide introduced at specific position from other nucleotides in the NA molecule) owing to differences between redox potentials of the DNA components and those of the labels (Fig. 1B) [3,5,17]. This is usually difficult without DNA labeling in the majority of natural target DNAs due to more or less even distribution of all four nucleobases in both complementary strands. In addition, using different tags for different nucleotide sequences makes it possible to analyze multiple targets in parallel ("multicolor" or "multipotential" NA labeling).

Comment: It is recommended to avoid using the term "marker" in the context of DNA labels due to possible confusion with clinical diagnostic markers.

Covalent labeling of NAs can in principle be conducted during chemical synthesis of oligonucleotides (often on commercial basis), by chemical modification of natural NAs or via enzymatic incorporation of modified nucleotides (available in the form of deoxynucleotide triphosphates, dNTPs) by primer extension or PCR (Table 1).

3.5.1 Electroactive groups

Besides reasons mentioned in the previous paragraph, the purpose of using electroactive groups attached to NA is analogous as mentioned above for the noncovalent indicators, i.e., to get analytically useful responses at relatively low overpotentials and to enable creation of reusable sensors based on labels undergoing reversible electrochemistry. Perhaps the most prominent electroactive DNA label has been ferrocene (Fc) tethered to the ends of synthetic ODNs or internally incorporated using Fc-labeled dNTPs and DNA polymerases [23]. Similar applications have been found by other electroactive groups, such as daunomycin, anthraquinone, thionine, bipyridine chelates of Ru or Os, nitrophenyl or aminophenyl groups, etc. Osmium tetraoxide complexes with nitrogen ligands (Os^{VIII},L) [24,25] or analogous osmate complexes (Os^{VI},L) [26] represent examples of electroactive tags attached to the natural NAs (or synthetic ODNs composed of natural nucleotides). Os^{VIII},L bind preferentially to thymine bases in ssDNA and have been applied for oligoT tail-labeling of ODN probes, while Os^{VI},L react primarily with *cis*-diols and are, in principle, suitable for the labeling of 3'-terminal ribose in ribo-

Table 1 Overview of detection techniques used in electrochemical DNA sensors.

Detection principle	Examples	Label-free[a]	Reagent-less[a]	Typical applications in sensors
Intrinsic NA electroactivity	Guanine oxidation at carbon-based electrodes	Yes	Yes (may be combined with redox mediators)	DNA hybridization, DNA damage
	Tensammetric DNA responses at mercury-based electrodes	Yes	Yes	DNA damage
Redox indicators	Electrostatic (anions or cations)	Yes	No	DNA hybridization, DNA damage host–guest interactions[b]
	Groove binders, intercalators	Yes	No	DNA hybridization, DNA damage, host–guest interactions[b]
Covalently bound redox labels (tracers)	Organometallics (Fc), metal chelates, organic moieties, nanoparticles	No	Yes (may be employed as redox mediators using a soluble depolarizer)	DNA hybridization, primer extension, DNA-mediated charge transfer, mismatch detection
Enzymes coupled to DNA	Phosphatases, peroxidases	No	No	DNA hybridization, PCR monitoring

[a]Label-free techniques use no chemical modification of NA probes, targets, or other analytes interacting with NA. Reagent-less techniques use no additional chemical reagents (indicators, redox mediators, enzyme substrates) to generate an analytical signal.

[b]The interacting small-molecule partners may feature redox indicators to analyze properties of the surface-confined DNA (e.g., DNA hybridization), or analytes to be determined via interaction with the DNA recognition layer (host–guest interactions).

nucleotides and RNAs. Many of the transition-metal-based electroactive tags are electrochemically "tunable" as their redox potentials can be influenced by choice or derivatization of the ligands [23,24]. In addition, some redox labels coupled to nucleobases (such as Fc) respond to the nucleobase type and/or to incorporation into DNA. Thus, the palette of NA redox markers offers many applications in DNA sensing which have been demonstrated and discussed in the literature.

3.5.2 Enzymes

In general, employment of the enzymes in biosensing is advantageous due to the inherent "biocatalytic" signal amplification (see Section 3.6.2) [5,17,27]. Enzymatic conversion of a substrate to a product which differs from the substrate by its electrochemical properties can serve for indirect electrochemical sensing of a molecular interaction. Alkaline phosphatase (ALP) and peroxidases belong to the most frequently used enzymes in the NA biosensors. The ALP possesses broad substrate specificity, being able to hydrolyze many phosphoesters (such as 1-naphthyl phosphate or *p*-aminophenyl phosphate whose dephosphorylated product can easily be determined at the carbon electrodes via irreversible or reversible electrooxidation, respectively). Peroxidases and oxidases producing hydrogen peroxide have usually been coupled to electrochemical (often amperometric) monitoring of H_2O_2 depletion or production. Typically, the enzymes are attached to NAs via biotin-avidin linkage, using enzyme-(strept)avidin conjugates and biotin-tagged NAs. The NA biotinylation can be attained via chemical ON synthesis or via (terminal or internal) introduction of biotinylated nucleotides by enzymes.

3.5.3 Nanoobjects

Metallic or semiconductor nanoparticles (nanocrystals, "quantum dots") have found many applications in both optical and electrochemical DNA sensing as unique, electronically tunable tools [5,28]. Nanoparticles or nanocrystals of gold, indium, zinc, cadmium, or lead chalcogenides and other materials have been used as labels covalently (often via thiol linkage) attached to DNA probes applied in amplified (see Section 3.6.2) DNA sensing. By combination of various nanoparticles (such as ZnS, CdS, and PbS), electrochemical "multicolor" DNA coding has been attained [28]. The nanoparticle tags have been applied in the classical biosensor concept (NA recognition layer-modified electrode) as well as in the magnetic bead-based approaches. The nanoparticle tracers were detected either in solid state after magnetic attraction of the beads bearing the hybridized DNA to an electrode surface (using "magneto-composite electrodes" or "magnetically switchable devices"), or by stripping voltammetric methods after dissolution of the nanoparticle material in a suitable solvent. Another popular type of nanoobjects used as DNA tags are carbon nanotubes (CNTs), which may be loaded with multiple nanoparticles or enzyme molecules, thus offering a considerable signal enhancement (see Section 3.6.2).

3.6 Specific features of the detection techniques

3.6.1 Signal-off vs. signal-on techniques

Electrochemical DNA biosensors produce two types of responses. The first is based on **appearance of a signal** (**signal-on**) resulting from a molecular interaction at the electrode surface. The signal-on techniques comprise, for example, the detection of strand breaks with mercury-based electrodes, hybridization sensors based on the guanine oxidation, covalently labeled NAs and noncovalent indicators associating preferentially with dsDNA, and sensor for host–guest interactions based on the electrochemical activity of the guest binders.

In the other group of techniques, **diminution of a measured signal** (**signal-off**) due to the interaction of interest is observed. These techniques include most of the sensors for DNA damage based on the guanine oxidation currents, hybridization sensors employing the indicators associating preferentially with ssDNA or based on anionic indicators, some types of electrochemical molecular beacons (see Section 4.1) and all competitive assays.

Comment: In general, the signal-on approaches can be expected to possess better analytical parameters than the signal-off ones. The reason lies in strong background responses in the signal-off techniques. When a decrease of an initially large signal is to be evaluated, change of the response (e.g., peak height) has to exceed standard deviation of the measurement, which limits sensitivity of the assay. For example, relatively large portions of the guanine residues in DNA have to be damaged or CP hybridized to observe reliable change (decrease) of the measured response. The signal-off techniques usually work well in model systems but may be less effective in analysis of real samples where, e.g., lower hybridization yields or relatively small portions of damaged guanines due to exposure to trace concentrations of genotoxic substances can be expected. In addition, the signal diminution may be caused by nonspecific destruction of the DNA recognition layer, which may result in false-positives hardly recognizable from specific responses of the sensor.

3.6.2 Signal amplification

Amplification of analytical signals is an important feature of NA biosensing because it is often desirable to detect a small amount of the analyte (specific nucleotide sequence, a point mutation, rare DNA lesions, etc.) in huge excesses of nonspecific NAs (other nucleotide sequences, intact DNA). Despite accumulation (enrichment) effects resulting from the biomolecular interactions themselves, as well as amplification of the genetic material to be analyzed by PCR, it is usually convenient to choose a (signal-on) detection technique providing enhancement of a response resulting from a single interaction event. This signal amplification can be attained by several ways [4,5,16,27,28], for example:

(a) employing multiple electrochemically active species in target DNA or signaling probes. These may be intrinsic components of the NA (e.g., guanine residues) or introduced labels (e.g., multiple redox-active tags used in the tail-labeling techniques [24]);
(b) using labels undergoing multi-electron electrochemistry or electrocatalytic processes providing high electron yields (e.g., Os,L at mercury-based electrodes [3,14]);
(c) employing biocatalysis (one molecule of the enzyme used as an NA tag can catalyze conversion of many substrate molecules into a detectable product [5,21,27]);
(d) in the nanoparticle-based sensing strategies (by tethering one nanoparticle per RP molecule, a large number of the trace atoms is collected per hybridization event; further signal enhancement can be attained by precipitation of additional tracer amount [28]);
(e) multilevel signal amplification has been achieved by application of different kinds of particles (microbeads) or nanoobjects such as CNTs, each carrying many redox marker entities (simple redox labels such as Fc, nanoparticles, or enzymes [28]); and
(f) in the mercury electrode-based sensors for DNA strand breaks, amplification of the signal is achieved through extensive surface denaturation of the DNA duplex around the lesion [16].

4. NUCLEIC ACID INTERACTIONS AND RELATED SENSORS

4.1 DNA hybridization and sequence-specific DNA sensing

DNA hybridization is based on the ability of ssDNA to form a DNA double helix (dsDNA) with its counterpart exhibiting a complementary nucleotide sequence. In DNA hybridization sensors, a specifically designed ssNA probe with a defined (known) nucleotide sequence is usually immobilized on a surface (in such a case, the NA probe is called **capture probe**, CP). The probe is used as a recognition element to test nucleotide sequence of **target DNA** (tDNA) in the sample solution. If tDNA contains a sequence complementary to the probe, hybrid dsDNA is formed [5,17,21]. This principle belongs to pivotal principles of the methodic arsenal used in modern molecular biology. Similar considerations can be applied to target RNA (tRNA). An NA biosensor is created by the immobilization of the probe onto

a transducer surface in a manner allowing the probe to interact with a target analyte under optimum conditions (pH, temperature, and ionic strength). Formation and stability of the hybrid depends upon the degree of complementarity (sequence matching) between the probe and target. By varying the pH, temperature, and the ionic strength conditions (hybridization stringency), the hybridization efficiency can be controlled to allow hybridization of probe-target pairs that are complementary, either full or partial, allowing the detection of single- or multi-base mismatches (see Section 4.1.4).

4.1.1 Detection techniques used in DNA hybridization sensors

Basic principles of the electrochemical detection approaches applicable in the DNA biosensors are overviewed in Section 3. Here, several examples of experimental arrangements typical for electrochemical DNA hybridization sensing are mentioned.

Label-free and indicator-less detection of target DNA typically uses guanine residues in the target DNA as the source of analytical signal. The guanine residues can be electrooxidized directly or using the redox mediators to achieve the oxidation of guanines not being in close contact with the electrode (see Section 3.3.1).

Comment: These approaches are inherently suitable for analyzing nucleotide sequences exhibiting considerable bias of guanine amount in one of the complementary strands (in fact, excess of guanines strand serves as a marker of the tDNA hybridized with G-poor CP). To achieve reliable distinction between CP and complementary tDNA in any nucleotide sequence, CPs in which guanine residues were replaced with hypoxanthines have been introduced.

Noncovalent redox indicators featured by diverse redox-active electrostatically interacting species, groove binders and DNA intercalators have been employed to distinguish between the ssCP (indicating no hybridization having taken place) and hybrid duplex at the electrode surface (indicating successful hybridization). These indicators can respond simply to the change of DNA amount (negative charge density) at the surface (electrostatic indicators) or can recognize DNA structure (groove binders or intercalators selectively binding to duplex DNA).

Sandwich hybridization assay employing a covalently labeled **reporter (signaling) probe** (RP) involves two NA-NA recognition steps (CP-tDNA, tDNA-RP), thus in principle improving the selectivity [5,17,21]. The RPs are designed to hybridize with the tDNA at a site next to the sequence recognized by the CP to confer efficient electronic communication between the label and the electrode.

Comment: Positioning of the RP close to the electrode surface is less critical when enzyme labels (producing soluble, diffusion-free indicators) are used or, in general, in the "double-surface" bioassays [5] (see Section 4.1.2).

Electrochemical molecular beacons, employing hairpin-forming probes, have been introduced as an analogy of optical molecular beacons in which the on-off switching of fluorescence is achieved by a change of conformation of a probe-bearing fluorophore at one of its ends and quencher at the other [29]. Electrochemical variants involve an ODN immobilized at the electrode by one end, labeled with a reversible redox marker (usually Fc) at the other. Within hairpin (stem-loop) structure of the probe, the label is located close to the electrode surface and yields a characteristic electrochemical response.

Comment: In the presence of complementary tDNA, a rigid linear duplex DNA is formed and the label is moved away from the electrode, resulting in elimination of the signal. The immobilized ODN need not necessarily form the stem-loop structure (which extends choice of target sequences detectable by the electrochemical molecular beacons), as differences in the flexibilities of the labeled ss probe and the hybrid duplex are sufficient to switch on/off the measured signal.

4.1.2 Primer extension-based sensors

The basic principle of DNA hybridization, i.e., probe–target pairing, can be combined with primer extension techniques [23,30]. An ODN probe with free 3'-hydroxy group hybridized to tDNA possessing ss 5' overhang can serve as a primer for in vitro DNA synthesis in the presence of a DNA polymerase and a mixture of deoxynucleotide triphosphates (dNTPs) on the target DNA template. When the dNTP mixture contains a labeled dNTP, the tag (or multiple tags) is introduced into the synthesized stretch, which can be utilized analytically. Because the newly synthesized DNA stretch is complementary to the tDNA overhang, this strategy not only allows one to indicate the probe (primer)-tDNA hybridization, but also to get information about the nucleotide sequence next to the probe–target hybrid (such as abundance of a particular nucleobase, detection of single nucleotide polymorphisms, etc.). The primer may be represented by a surface-attached CP; thus, primer extension can be performed at the electrode surface.

4.1.3 Detection of mutations and sequence polymorphisms

Detection of **mutations** (hereditable alterations in the genomic nucleotide sequence, such as substitutions of single base pairs, insertions or deletions of base pairs, or longer DNA stretches) is an important task due to its close connection with the genome function and pathogenesis of severe diseases. Electrochemical techniques used for the detection of **single nucleotide polymorphisms** (SNPs, point mutations) include several approaches, some of which are analogous to those applied in connection with the other detection techniques (such as fluorescence) [16,17,21]. One principle is based on different stabilities of duplexes that are fully complementary between the probe and tDNA (homoduplexes between wild-type probe and wild-type tDNA or mutant probe and mutant target) and those involving mismatched nucleotides (heteroduplexes between wild-type probe and mutant target or vice versa). Discrimination of perfectly matched and mismatched duplexes can be achieved by performing DNA hybridization at stringent conditions achieved by elevated temperature, decreased ionic strength, or via applying PNA probe instead of DNA. Under optimum conditions, the homoduplex gives positive hybridization response while the heteroduplex is not stable, thus giving a signal-off response to the mutation in one of the hybridizing strands.

Another generally applicable technique relies on primer extension incorporation of a labeled nucleotide within the SNP site [23,27]. The target template is annealed with a primer complementary to the target segment "upstream" (relative to DNA polymerase-catalyzed elongation of the primer, which always proceeds in the 5' $\rightarrow$ 3' direction) to the position of interest, and a labeled dNTP (e.g., with biotin to attach an enzyme in the following step, or with a redox marker) is added to the reaction mixture. Under proper conditions, the labeled nucleotide is attached to the primer only when it is complementary to the base at first "free" position. Using different labels for different nucleotides, all four possible bases within the SNP site can be probed in a single reaction. These approaches have successfully been applied in both classical DNA biosensors and the alternative magnetic bead-based assays [5].

Other electrochemical sensors designed for the SNP detection utilize electronic properties of the duplex DNA and perturbations in the DNA electronic properties in the presence of single-base mismatches [31]. Disruption of the π-stacks within the DNA double helix due to presence of the mismatch has been shown to prevent DNA-mediated charge transfer between electrode and an intercalator bound at the opposite (relative to the electrode surface) end of the double helix, which was efficient in the perfectly matched (and perfectly base pair-stacked) homoduplex. Analogous principle was applied in sensing interactions of surface-attached DNA duplexes with proteins causing DNA bending and/or base flipping (for DNA–protein interactions, see Section 4.2.2).

Another important class of genomic mutations comprises expansion of the lengths of trinucleotide repeat sequences. Electrochemical determination of the length of guanine-containing triplet repeats was achieved by the mediator-based guanine electrocatalytic oxidation technique (see Section 3.3.1) combined with radioactive labeling. Other approaches applied for this purpose involve multiple

hybridization of a labeled RP spanning several triplet units with the expanded triplet repeat [5,17]. The number of RP molecules hybridized (or labels collected) per the tDNA strand is proportional to the length of the repetitive sequence, which is—after proper normalization to the number of target strands—reflected by intensity of the measured signal.

4.2 Other association interactions

4.2.1 Nucleic acid interactions with low-molecular-mass compounds

Three **main binding modes** are recognized as noncovalent NA association host–guest interactions [16,17]:

(a) intercalation between the stacked base pairs of dsDNA,
(b) binding at major or minor grooves of the DNA double helix, and
(c) electrostatic interactions.

Note: The detection of NA (DNA) association with low-molecular-mass compounds like drugs and chemicals represents an important aspect of studies in drug discovery and environmental processes. NA biosensors serve as effective screening tools for in vitro tests of NA interactions. Such tests are also of importance for the choice of the NA indicators. Due to the preconcentration effect within NA structure, specific (not selective) analytical detection/determination of a trace low-molecular-mass analyte or group of analytes could also be a result of such a study.

The **intercalation** represents an insertion of guest molecules between the stacked base pairs of the double-helix structure. It typically occurs at compounds of a planar structure with 3–4 aromatic rings. To accommodate an intercalating molecule, the dsDNA chain must lengthen and unwind slightly. Thus, intercalation can cause a lengthening of the DNA helix and perturbation of the phosphate backbone. This can in turn lead to a long-range deformation of the DNA helix altering the structure and functionality of the molecule. The amount of intercalating molecules depends both on the NA primary sequence and intercalator nature. For example, MB is intercalated primarily in guanine reach parts of dsDNA to the average amount of one molecule per 3–4 base pairs (bps).

Comment: While some intercalators (e.g., doxorubicine, 1,10-phenanthroline complexes of transient metals, or Fc naphthalene diimide) retain their electrochemical activity after the intercalation, some other, e.g., phenothiazines, do not show significant current signals after intercalation. The initial step of intercalation can result in secondary interactions which can be used for the detection, e.g., electron transfer from guanine residues (e.g., using the $[Ru(bpy)_2]^{2+}$ complex) or generation of oxygen reactive species able to initiate oxidative cleavage of ribose cycles in the primary DNA sequence.

Major and minor groove binding molecules bind to the exterior of the grooves of dsDNA. Whereas the intercalating molecules tend to contain fused aromatic heterocycles, the minor groove binders tend to be unfused aromatic heterocycles. Typical groove binding analyte (a drug) is a flat crescent-moon-shaped molecule that holds itself in the groove through hydrogen-bonding and van der Waals interactions.

Electrostatic interactions are formed between positively charged guest molecules and the negatively charged DNA sugar-phosphate backbone.

Comment: Depending on reaction conditions, these modes can be combined. For instance, the dsDNA interaction with positively charged metal complex compounds with aromatic ligands is predominantly electrostatic at low ionic strength and predominantly intercalative one at high ionic strength. A predominant character of the binding interaction of the components of electrically charged redox pairs (e.g., Co^{III}/Co^{II}, Fe^{III}/Fe^{II}, etc.)

can be estimated from a net negative or positive formal potential shift when the first one indicates the stabilization of the component in a higher oxidation state over that in a lower oxidation state, i.e., the electrostatic interaction and the second one can be ascribed to the intercalation [17,32]. As a result of the association interaction studies, specific parameters such as NA binding site, binding site size, etc. used to be obtained.

Note: Some compounds, particularly from the drug family (e.g., mitomycin C), form **covalent bonds** with NA bases to create **adducts** yielding specific electrochemical responses [16]. Some of these compounds (e.g., cisplatin) are used either to add substituents onto base residues or to form cross-links between different sections of DNA or between DNA and proteins.

For the **detection of the association interactions**, typically, the NA-modified electrode is exposed to the analyte solution, and, after allowing the interaction to take place on the surface, the electrochemical measurement is performed directly in the analyte solution or after the biosensor transfer into blank supporting electrolyte (a buffer solution). The electrochemical measurement itself is based on the monitoring of the responses related either to an electrochemically active analyte or to the guanine and 8-oxoguanine—a product of the guanine oxidation promoted by internal electron transfer with the participation of the analyte included in the complex with NA.

Comments: In both cases, changes in the responses measured prior to and after the contact of NA sensor with an analyte are considered. The direction and degree of the signal shift depend on the mechanism of interactions taking place onto the electrode surface. Thus, full intercalation of an analyte results in sufficient suppression of its signal. Partial intercalation or the coordination of the analyte molecule on the NA surface can lead to either decay of the analyte signal which is commonly much milder than that in the previous case or even an increase in the analyte current, meaning preferable coordination of the NA-analyte complex that promotes the electron transfer. In addition to the above changes of the analyte response, simultaneous shifts of the guanine (8-oxoguanine) voltammetric peak are often observed [16]. This can be taken as an independent evidence for the NA–analyte interaction and the role of NA altering the analyte signal.

The distortion of the surface DNA layer can also be specified by appropriate changes in the resistance of the charge transfer and capacity of the surface layer measured by EIS. Impedimetric measurements also provide possibility to detect electrochemically inactive analytes which do not exert remarkable changes in the guanine oxidation current [33].

Besides the net formal potential shift of a redox pair of the guest molecule, a competitive effect of another intercalator (electroactive one in the case of non-electroactive guest analyte under investigation) and an effect of the medium ionic strength may indicate type of the interaction.

4.2.2 Nucleic acid interactions with proteins

The NA biosensors can be applied in studies of NA–protein interactions in two ways. The first one is suitable for detecting **catalytic activities of NA-processing enzymes** such as nucleases, ligases, or polymerases. Examples of the applications of the DNA sensors based on the NA enzymatic conversions (which are always preceded by physical interaction between the NA and the protein) are mentioned in Sections 4.3.1 and 4.3.6. The other group concerns **affinity interactions of proteins** (which can but need not be enzymes) with the NAs.

Affinity biosensors for DNA–protein interactions can in principle employ analogous detection techniques as mentioned above for DNA hybridization sensors. Proteins are electroactive owing to the

presence of electrochemically active amino acid residues, allowing them to be detected electrochemically without any labeling. For example, interaction of *E. coli* ss binding protein with DNA immobilized at single-walled CNTs modified screen-printed carbon electrodes was detected using current responses due to electrochemical oxidation of tyrosine and tryptophan residues in the protein [34].

In specific cases, binding of a protein to dsDNA can disturb base pair stacking via flipping-out a nucleobase or via bending the duplex. These perturbations can affect the dsDNA-mediated charge transfer at a gold electrode (see also Section 4.1.3) [31], as reported for, e.g., a base-flipping enzyme MHhaI. On the other hand, duplex DNA was reported to conduct electrons between the electrode and [4Fe-4S] cluster in a DNA repair protein MutY, allowing detection of this protein binding to dsDNA anchored at the electrode surface.

4.2.3 Aptamer–target interaction

The design for aptamer-based sensor (or aptasensor) largely relies on the inherently different recognition modes of each aptamer-target complex. Generally, aptamers incorporate small molecules into their NA structure, leaving little room for the interaction with a second molecule. Thus, small molecules are typically detected by a single-region binding assay. By contrast, protein targets are structurally complicated, allowing the interplay of various discriminatory contacts. As a result, protein can be assayed via both single-region binding and dual-region binding assay (sandwich assay).

The majority of the detection principles described in Section 2 are applicable to electrochemical aptasensors. Label-free modes and modes free of reagents are based upon the change in electrode surface behavior after the formation of the aptamer-target complex (generally monitored by EIS or FET) or upon the evaluation of the target properties (i.e., intrinsic electrochemical responses of the protein). Different label modes are possible. Redox-active compounds can be covalently tethered to an aptamer or bound to an aptamer complementary sequence (which modulate the indicator signal upon the formation of aptamer-target complex), as well as present as indicators in the solution phase. Sandwich assay with a secondary aptamer (or an antibody) labeled with enzymes, metal nanoparticles, etc., as well as methods based on the activity of the protein (in the case this protein is an enzyme) are other examples.

4.3 DNA damage

The term "**DNA damage**" refers to any alteration in the chemical structure of the genetic material resulting from interactions with physical or chemical agents occurring in the environment, generated in the organisms as by-products of metabolism or used as therapeutics [16]. The main types of DNA damage include interruptions of the sugar-phosphate backbone (strand breaks), release of bases due to hydrolysis of *N*-glycosyl bonds (resulting in abasic sites) and a variety of nucleobase lesions (adducts) resulting from reactions of DNA with a broad range of oxidants, alkylating agents, etc. DNA damage may affect crucial cellular functions and can, when unrepaired, give rise to mutations.

Comment: Terms "(product of) DNA damage" (lesion, adduct…) and "mutation" should not be intermingled. **Mutations** refer to changes in DNA sequence—substitutions, deletions, or insertions of (one or more) base pairs; hence, mutated DNA sequences contain standard base pairs that are perfectly complementary (and from this point of view are not damaged) but carry hereditably (irreversibly) altered genetic information. On the other hand, in **damaged DNA** the chemical nature of individual nucleotides is changed, which can result in mutations via repeated replication of the damaged DNA, but the genetic information can still be preserved in the complementary strands, allowing proper DNA repair prior to the replication. In addition to changes of covalent bonds, the term "DNA damage" is sometimes extended to biological function-affecting alterations of DNA structure induced by noncovalent binders such as intercalators (see Section 4.2).

Altered chemical, physico-chemical, and structural properties of damaged DNA are reflected in its behavior at the electrode, which has been utilized in numerous techniques designed for **DNA damage detection**. Electrochemical biosensors have been used not only to detect, but also to induce and control DNA damage at the electrode surface via electrochemical generation of the damaging (usually radical) species [16,35].

4.3.1 Label-free detection of strand breaks with mercury-based nucleic acid sensors

Electrochemical behavior of DNA at the mercury-based electrodes is strongly influenced by its backbone structure, allowing a perfect discrimination between DNA molecules containing or lacking free ends. Owing to the potential induced surface denaturation of DNA double helix (see Section 3.2.1), DNAs with free ends produce under certain conditions electrochemical responses specific for ssDNA (which are not produced by intact dsDNA). Denaturation of closed circular DNAs is prevented for topological reasons. This variation in electrochemical behavior has been utilized for the sensitive detection (allowing us to recognize one lesion among ~10^5 intact nucleotides) of breakage to the DNA sugar-phosphate backbone.

Mercury and amalgam electrodes chemically modified with an adsorbed layer of super-coiled plasmid DNA have been used to monitor nicking of super-coiled plasmid DNA with enzymes (such as DNase I) as well as reactive radical species that destroy the deoxyribose moieties. The same principle can be used for indirect detection of some types of nucleobase lesions after their conversion to strand breaks by specific enzymes, as well as for monitoring of a reverse process, i.e., the repair of strand breaks by action of the DNA ligases.

4.3.2 Redox indicator-based sensing of DNA degradation with carbon-based nucleic acid sensors

A redox indicator-based sensor was designed to detect DNA degradation by chemical systems producing reactive oxygen species. The technique employs a metal complex like $[Co(phen)_3]^{3+}$ binding to dsDNA at the electrode surface. Interaction of the indicator with intact dsDNA results in enhancement of its voltammetric signals. Degradation of DNA results in diminution of the indicator voltammetric peak depending on the degree of DNA damage. The magnitude of peak current decrease represents the response to the DNA damage. This type of sensor was also applied in studies of anti-oxidative properties of various natural substances preserving DNA from its damage [17].

4.3.3 Sensors based on guanine redox processes

Techniques based on measurements of intrinsic responses due to the guanine residues belong to the most frequently applied techniques in the DNA biosensors due to (i) well-defined guanine responses at carbon as well as mercury-based electrodes, and (ii) the fact that guanine is, among the DNA bases, the most frequent target for a broad range of genotoxic agents [16,35]. Due to chemically or photochemically induced chemical changes in the guanine moiety, its electrochemical features may be altered and responses corresponding to the parent base lost. Thus, decrease of the guanine peak height relative to its intensity yielded by intact DNA represents the response to damage to the nucleobase. Since natural DNAs contain many guanine residues, partial decrease of the guanine peaks is usually observed, depending on the extent of the DNA damage. Decrease of the guanine redox peaks of DNA is obviously also caused by a release of the base from the polynucleotide chains, an event often following modifications within the guanine imidazole ring.

Comment: This mode of the DNA damage detection is sometimes even more sensitive to the concentration/effect of DNA damaging species than other techniques used at the DNA electrochemical biosensors. Nevertheless, the guanine oxidation current was also reported first to increase and then to decrease, indicating more complex DNA damage profile like helix opening and then degradation.

4.3.4 Electroactive products of DNA damage

Some of the products of DNA damage exhibit characteristic electrochemical activity. For example, 8-oxoguanine (8-OG) is electrochemically oxidized at carbon electrodes at a potential significantly less positive than the parent guanine base (Fig. 1A) [16,17,35]. This feature has been utilized for determination of 8-OG in the presence of G, either via direct, or mediated electrochemical oxidation (using $Os^{III/II}$ complexes as mediators).

Note: The damaged (modified) DNA may also acquire specific electrochemical features from newly introduced moieties forming stable adducts with the nucleobases (e.g., mitomycin C and other electroactive drugs whose pharmacological effects involve DNA modification).

4.3.5 Layered assemblies for genotoxicity screening

Multilayer assemblies of cationic redox-active cationic polymer films, DNA, and heme proteins at carbon electrodes were designed for testing genotoxic activity of various chemicals [36]. In these devices, layers of the enzymatically active hemoproteins mimic metabolic carcinogen activation processes (e.g., styrene is enzymatically converted to styrene oxide). The activated species diffuse into the DNA layer, where attack guanine residues. Consequent "unravelling" of the DNA double helix facilitates electrocatalytic oxidation of other guanine residues mediated by a ruthenium complex immobilized in the cationic polymer film.

4.3.6 Molecular beacon-like sensor for nuclease and ligase activities

An electrochemical biosensor based on a hairpin DNA probe labeled with Fc (analogous to the molecular beacon mentioned in Section 4.1.1) was reported for monitoring the activities of nucleases (generating ss breaks) or DNA ligases (sealing the break) [37]. The stem (duplex) part of the hairpin structure contained a ss break and the Fc-labeled segment was removed under denaturing conditions. In the presence of the ligase activity, the break was joined, preventing removal of the Fc-labeled segment and resulting in appearance of a current signal due to the Fc oxidation. When the continuous form of the hairpin (without the break) was exposed to a restriction nuclease, the same procedure resulted in diminution of the current signal.

5. CONSTRUCTION OF BIOSENSORS

5.1 Transducers

The way of NA biosensor preparation is of great practical importance. It depends on the biosensor particular use and determines its performance parameters. Mercury [or mercury film (MFE)] and carbon (glassy carbon, carbon paste, graphite, graphite-epoxy composite) electrodes, as well as some other transducer materials such as gold, indium tin oxide (ITO), and solid amalgam electrodes (SAEs) belong to the most popular transducers in the NA biosensor preparation.

Comment: The term "substrate" for the transducer material should be avoided because it is used typically in description of enzymatic reactions. Attention has to be paid to gold and other electrodes often used for electrochemical NA sensing and not as the biosensor transducers.

Besides bulk electrodes mentioned above, various thin- and thick-film electrodes deposited on an insulating material were suggested as mass produced, and, therefore, reproducible and inexpensive strip solid transducers [38,39]. The two most common materials used for the fabrication of film electrodes are gold and carbon. The gold thin-film electrodes are prepared by sputtering and vacuum evaporation leading to continuous gold films. Gold arrays can be obtained by a combination of these methods with microlithography. Thick-film electrodes are typically prepared by using screen-printing technology as screen-printed electrodes (SPEs), mostly screen-printed carbon electrodes (SPCEs).

The choice of the electrode material is connected, on one hand, with the electrochemical process of interest. Mercury and carbon electrodes are of interest at the investigation of intrinsic NA responses. Mercury electrodes and some SAEs exhibiting high hydrogen overvoltage can operate at relatively high negative potentials. The potential windows of most of the solid electrodes are shifted by approximately 1 V to more positive values compared to the mercury-containing electrodes. The solid electrode are thus typically suitable for studies of the NA oxidation processes, while mercury electrodes (both liquid and solid) are better suited for the NA reduction. On the second hand, the electrode material used is also closely related to the choice of the NA immobilization technique.

Fully electrical biochip technology in this field is represented by **DNA array sensors** made in silicon technology [40]. At a low-density chip, the transducer is realized in several array positions (e.g., of 0.5-mm diameter) with inter-digitated electrode (e.g., of 800-nm width separated by 400-nm-wide gaps) [41]. Such microarrays can be freely designed for the particular use. For instance, alkanethiol-modified capture ODNs were attached to the gold surface and viral target DNA obtained by the PCR amplification was detected using hybridization event. Internal standards can be immobilized as well. Enzyme label can be introduced through the PCR primer and redox recycling of the enzymatic reaction product between the ultra microelectrodes is used to enhance the signal.

Label-free detection can be achieved by monitoring a change in conductance or resistance and capacitance between neighboring electrodes, for instance, through the hybridization. Amino- or thiol-modified ssNA probes can be immobilized covalently on the gap between the electrodes (and not on the electrodes) by using derivatized trimethoxysilane linkers. Difficulties of the frequency-dependent impedance method in low-frequency region can be overcome with transient techniques.

Nanotechnology-enabled sensors are already widely used in the field of biosensors including NA-based sensors [42–44]. Gold nanoparticles [28] and carbon nanomaterials, particularly CNTs [45], have attracted attention due to their unique structural, electronic, mechanical, and chemical properties. The inherent electroactivity and effective electrode surface area of CNTs lead to a large enhancement of the current responses, compared to those obtained at conventional carbon electrodes. Moreover, CNTs can self-organize with DNA molecules. The mixed layer formed keeps stability of the surface coverage and can be used as a new electronic material, for example, to impart electrochemical properties of some proteins. An ability of DNA assembled on nanotubes to interact with drug molecules can facilitate the construction of new types of miniature DNA biosensors.

5.2 Nucleic acid immobilization

After the transducer choice, **NA immobilization** on the electrode surface is an initial step that plays a major role in the overall sensor performance. At this step, experimental conditions have to be optimized for each special application. For this purpose, a large spectrum of methods typically used at biosensors can be utilized.

5.2.1 Noncovalent binding

The NA adsorption on a transducer immersed into dilute NA solution (to create thin NA films), as well as an evaporation of small volumes of the NA solution to dryness (to create thicker NA layers) are typically used as ways of the NA **physisorption**. This binding is quite strong particularly at the mercury and some carbon electrodes and may involve, depending on the NA structure and electrode surface charge, hydrophobic and/or electrostatic interactions of the NA bases and negatively charged sugar-phosphate backbone, respectively [3].

Comment: For the carbon paste electrode (CPE) and, sometimes, for SPCE, a pre-activation by anodic polarization at +1.7 V vs. Ag/AgCl for some time (several minutes) was suggested [17,46]. Such anodic pre-activation improves the stability of physisorbed DNA layer even though the adsorption is performed in open-circuit mode after such electrode treatment. Adsorption can be performed at controlled potential. However, si-

multaneous electroactivation of the transducer and immobilization of NA performed typically in the NA solution could represent a risk of NA oxidative changes.

At the direct adsorption, an accessibility of the immobilized DNA by a guest molecule or another ssDNA is generally limited due to the contact of the NA backbone with the electrode surface. This results in poor detection efficiency of the hybridization.

Note: Surfactants such as cetyltrimethylammonium bromide adsorbed on the hydrophobic surface of CPE were shown not only to improve electrochemical properties of the transducer but also to be a material for the immobilization of dsDNA [47].

Experimental conditions have a strong effect on the adsorption of oligo- and poly-nucleotides on the untreated glassy carbon electrode (GCE) [48]. Contrary to CPE, a negative effect of the potentiostatic and air-oxidative pretreatment of the electrode surface was observed together with no effect of the accumulation potential. Hence, there is a small contribution of the negatively charged phosphate backbone to the NA adsorption at this electrode. The confined DNA layer is stable on air at room temperature and the adsorption is strong enough to perform the measurement after the electrode transfer into blank solution. The stability of the layer in solution depends on the quality of buffer and time. During the ss- and dsDNA spontaneous adsorption on a highly oriented pyrolytic graphite electrode (HOPG), DNA condenses, forming complex network films with pores exposing the HOPG surface [49].

Note: Thin DNA films formed in pH 5.3 acetate buffer exhibit better coverage of the electrode surface with DNA molecules than the films formed in pH 7.0 phosphate buffer solutions. The application of a positive potential during the adsorption step enhanced the robustness and stability of the DNA films with the formation of bigger network holes and a more condensed and compact self-assembled DNA lattice [49].

An **entrapment within polymeric films** enables more stable immobilization and is of special interest for the genosensors and all-electronic microarrays. Cationic and conducting polymers are used where the last ones have an advantage of electronic conductivity or electrochemical addressability. Various ways of immobilization of DNA and oligonucleotides anions were used, including polyelectrolyte interaction owing cationic groups of the polymeric film, physical entrapment, and others utilizing previously deposited polymer films, electropolymerization of a monomer-modified NA, copolymerization of monomer and NA, etc. [50,51].

Comments: NA binding based on electrostatic physisorption exhibits advantages such as simplicity and mild conditions of immobilization together with accessibility of the immobilized DNA. On the other hand, adsorption of short ODNs is not stable and could be done within the polymer host. Entrapment of NA in the bulk of a polymer can also result in high loading. However, its conformational mobility could be restricted. At the modification of microelectrodes, the polymerization reaction should be initiated electrochemically, allowing the selective modification of individual electrode elements in an array.

NAs were also entrapped within hybrids formed by the **sol–gel techniques** which combined the biomolecule with inorganic materials [52].

Note: Simple and cheap noncovalent NA immobilization procedures are of interest particularly for disposable devices for routine and field use.

5.2.2 Affinity binding

Extremely strong **avidin-biotin system** is also often used to immobilize NA biotinylated at its 5' end by using avidin attached directly to carbon-based transducers [53]. These schemes have numerous variations depending on the way of avidin attachment. For example, it can be physically adsorbed, attached

via another biotin molecule, included in carbon paste, cross-linked with glutaraldehyde, incorporated in hydrogel, etc.

Comment: Avidin layer can be an inhibiting barrier for redox indicators. Utilization of avidin incorporated into redox polymer could lead to prompt immobilization with higher hybridization efficiency.

5.2.3 Chemisorption

Formation of **self-assembled monolayers** (SAMs) of thiols and other sulfur-containing derivatives of ODNs and PNAs on gold (also silver, platinum, palladium, iron, mercury, and other) transducers are also commonly used [54,55]. Typically, mixed monolayers of the 5'-thiol-derivatized NA and commercially available short-chain alkanethiols are formed where the adsorbed alkanethiol minimizes unwanted nonspecific binding of nonderivatized NA. Thus, chemisorption enables relatively strong and simple single-point attachment of the NA probe preserving its conformational mobility.

Comments: The abbreviation SAM for self-assembled monolayer is the same as used in the literature for S-AdenosylMethionine (SAM).

The thiol-modification of NA is rather tedious, and the yield is quite low. Nonspecific binding of NA strands on gold surface may represent another problem for the thiol-labeled NA which may be solved using mixed SAMs incorporating alkanethiols [39]. Relatively little is still known about how the NA-transducer connection affects the film integrity. Regarding this, DNA molecules with thiol-terminated linkers containing either ethane, hexane, or xylene spacers were used for the formation of densely packed monolayers on gold electrodes [56]. The dsDNA immobilization on gold can be controlled by electric potential. For instance, faster formation of a more compact layer of the thiolated ssDNA was achieved under application of low positive potential (+0.2 V vs. SCE) [57].

5.2.4 Covalent binding

Procedures involving the immobilization via one end of the NA molecule without damage to bases are recommended in literature to preserve the original DNA recognition ability/specificity and thus hybridization efficiency, and to obtain benefits of long-lasting and reusable immobilization. The NA immobilization can be well controlled and molecules nonspecifically and weakly bound to the surface can be removed.

The **carbodiimide method** has been widely reported. It is based on the fixation of chemically modified ODNs (e.g., amino-linked ODNs) onto activated electrode surface bearing oxidized (e.g., carboxylate) groups [58].

Chemically grafted NAs (e.g., amino end-modified NA) can be **covalently attached to the synthetic polymer films** bearing reactive linkers such as biotin, complexation ligand, etc. Click chemistry-based DNA immobilization represents another promising strategy [59].

Comment: The reaction with amino groups of nucleobases is not always fully considered in the studies.

6. SPECIFIC PERFORMANCE CRITERIA

As for chemical sensors and biosensors generally, at the NA-based biosensors there are also criteria for an evaluation of how the performance requirements are fulfilled for their particular application. Some of them characterize the NA biosensor and others the detection procedure used with the biosensor. The characteristics of the sensor are also classified as either static or dynamic [44]. Static characteristics are those that can be measured after all transient effects have stabilized to their final or steady state. They address the question of how much the sensor output changes in response to the input, etc. Dynamic

characteristics describe the sensor's transient properties, e.g., what rate is the output changing in response to the input, etc.

Many of the electrochemical NA biosensor performance criteria are influenced strongly by its building parts (type and amount of NA, type and pretreatment of the electrode), NA immobilization procedure, NA biosensor pretreatment, electrochemical detection mode, and so on.

Specific criteria of the NA-based biosensor are represented by the response time, storage time, and lifetime stability under specific experimental conditions, its biocompatibility, portable size, etc. Reproducibility of the biosensor fabrication, particularly when mass-produced transducers (such as SPCE) are used, is an important attribute that characterizes strip-to-strip properties of the transducer itself as well as the whole NA biosensor. The repeatability of the measurement with NA biosensor characterizes its ability to be used repeatedly within a given measurement/regeneration detection scheme. On the other hand, many NA biosensors are designed as single-use (disposable) devices. In some cases, irreversibility of the analyte-NA interaction (e.g., covalent NA damage) or of the electrode process giving rise to the response (e.g., guanine oxidation) precludes reusability of the biosensor.

Specific criteria of an analytical procedure used with the NA biosensor are represented by specificity/selectivity (i.e., recognition ability toward an analyte or group of analytes; see also Section 1), calibration curve parameters (limits of detection and determination, dynamic concentration range, and sensitivity) as well as trueness and accuracy. Some of them depend on accessibility of the immobilized NA for the interaction with an analyte. For instance, multiple contact sites of ss probe with the transducer may diminish the hybridization efficiency. The way of the sensor calibration is dictated by its reusability (see above) and is performed either conventionally or using different single-usable device(s)—one for detection and (an)other(s) for calibration.

Effects of experimental conditions such as pH, ionic strength, and temperature changes on these parameters should be evaluated as well. Sample size and sample pretreatment also need to be considered.

Comment: These data are often more or less absent in original studies or they are expressed in different ways to be compared. Commercial development of the DNA biosensors could also be a reason for it. Nevertheless, the biosensor characteristics (as complete as possible) are of great importance for both their utilization and further development.

7. CONCLUSIONS

A unique feature of the NA-based sensors is that they detect specific interactions of the NA recognition layer with substances of interest. They represent a faster, cheaper, and simpler alternative to tradition assays such as gel electrophoresis or membrane blots. Modern electrochemical NA biosensors also offer remarkable sensitivity and compatibility with micro-fabrication technologies. Electrochemical DNA chips constitute a compact device with good cost performance. Hybridization genosensors and all-electronic microarrays are of particular interest as they are expected to play a significant role in the future diagnostic market. Here, principles and ways have been shown how direct electrical reading of NA interactions is promising for development of simple and user-friendly NA sensing devices. Their critical evaluation is presented together with a comparison to other procedures of electrochemical NA biosensing.

LIST OF ABBREVIATIONS

A	adenine
AC	alternating current
bp	base pair
C	cytosine

CNT	carbon nanotube
CP	capture probe
CPE	carbon paste electrode
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
ds	double-stranded
dsDNA	double-stranded DNA
EIS	electrochemical impedance spectroscopy
Fc	ferrocene
FET	field effect transistor
G	guanine
HMDE	hanging mercury drop electrode
HOPG	highly oriented pyrolytic graphite electrode
ITO	indium tin oxide
K_d	dissociation constant
L	ligand
LNA	locked nucleic acid
MB	methylene blue
MFE	mercury film electrode
NA	nucleic acid
ODN	oligodeoxyribonucleotide
8-OG	8-oxoguanine
PCR	polymerase chain reaction
PNA	peptide nucleic acid
RNA	ribonucleic acid
RP	reporter probe
SAE	solid amalgam electrode
SCE	saturated calomel electrode
SELEX	systematic evolution of ligands by exponential enrichment
SNP	single-nucleotide polymorphism
SPCE	screen-printed carbon electrode
SPE	screen-printed electrode
ss	single-stranded
ssCP	single-stranded capture probe
ssDNA	single-stranded DNA
T	thymine
tDNA	target DNA

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