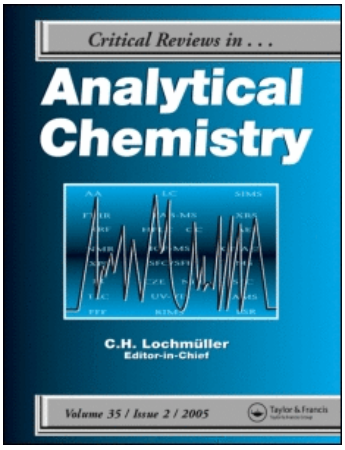

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

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Online publication date: 03 June 2010

To cite this Article Paleček, Emil and Jelen, František(2002) 'Electrochemistry of Nucleic Acids and Development of DNA Sensors', Critical Reviews in Analytical Chemistry, 32: 3, 261 — 270
To link to this Article: DOI: 10.1080/10408340290765560
URL: http://dx.doi.org/10.1080/10408340290765560

Critical Reviews in Analytical Chemistry, 32(3):261–270 (2002)

Electrochemistry of Nucleic Acids and Development of DNA Sensors

Emil Paleček and František Jelen

Institute of Biophysics of the Academy of Sciences of the Czech Republic, Královopolská 135, CZ 612 65 BRNO, Czech Republic

KEY WORDS: DNA damage detection, DNA hybridization at magnetic beads, electrochemical DNA sensors, nucleic acid electrochemistry, DNA at electrode surfaces, decentralized DNA diagnostics.

I. INTRODUCTION

In recent years our knowledge regarding the nucleotide sequences of a number of genomes has increased tremendously. With the completion of an increasing number of genomic sequences attention is currently focused on how the sequence data might be interpreted in terms of the structure, function, and control of biological systems. It now appears that methods of electrochemical analysis may find application in this exciting research area.

A. The Human Genome Project

Sequencing of the human genome is now in its concluding stage. Gene sequence data alone appear of little clinical use unless directly linked to sickness relevance. To test individuals for details in nucleotide sequences of their genomes and for expression of different genes new technologies are necessary. The most promising are DNA-based biochips. DNA chips are used at present for two types of analysis: (1) detection of mutations in specific genes as diagnostic markers; (2) the detection of differences in gene expression levels in health and disease. They are becoming important diagnostic tools and provide important information creating basis for new therapeutic approaches. These chips are already commercially available, produced by a number companies, including Affymetrix, Hyseq and Caliper Lab Chips; the latter company uses microfludic technology to manipulate minute volumes of liquids on chips. The detection of the nucleotide sequences in commercially available DNA chips has been based on fluorescence. At present, it is believed that electrochemical detection might be a better alternative.

II. ELECTROCHEMISTRY OF NUCLEIC ACIDS

At present, nucleic acids (NAs) electrochemistry is a booming field because the expectations of the development of electrochemical transducer-based devices for DNA damage detection and determining nucleotide sequences in DNAs and RNAs are high.[1-5] Efficient and inexpensive devices for determining RNA and DNA sequences are required for a better decentralized diagnosing, preventing and treating of many human diseases. Such devices will also be of use in other applications such as veterinary and forensic medicine, environmental testing, etc. The main advantages of electrochemical devices are their low-cost, simple design, fast response, low power requirements, small dimensions, etc.

A. History

In 1958 it was found by one of us (E.P.) that DNA and RNA are electroactive producing reduction and oxidation signals after interaction with electrodes.[6,7] It was the so-called oscillographic

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polarography at controlled a.c.[8] (constant a.c. chronopotentiometry according to the present nomenclature) that proved to be best suited for the analysis of NAs in that time. In 1958 it was believed that among the nucleic acid components adenine is polarographically reducible only at highly acidic pHs,[9] while other components are inactive. We showed that in addition to adenine (A) also cytosine (C) was reduced at DME at neutral pH[7,10] and guanine (G) produced a specific anodic signal, later explained by the oxidation of the guanine reduction product formed at highly negative potentials.[6,7, 11,12] In addition, all NA bases produced anodic signals due to the formation of sparingly soluble compounds with the electrode mercury,[6,11] which were later utilized for the cathodic stripping voltammetric determination of NA bases at nanomolar concentrations.[13-16]

Signals of adenine, cytosine, and guanine were produced not only by free bases but also by single stranded DNAs and RNAs (reviewed in Refs. 17-19). Soon it became clear that the electrochemical signals of DNA obtained with mercury electrodes were very sensitive to the DNA structure in solution.[17,18] In the 1960s and first half of the 1970s the oscillographic polarography and later the differential pulse polarography (DPP) produced early evidence of DNA premelting and polymorphy of the DNA double helix (reviewed in Ref. 20). The polymorphy of the DNA structure was later well established by the X-ray crystallography and NMR.[21] The first 2 decades of the electrochemical studies of nucleic acids (predominantly with mercury electrodes) were reviewed thoroughly.[18]

B. Adsorptive Stripping Voltammetry and DNA-Modified Electrodes

In the first half of the 1980s the use of electrochemical methods in biochemical and molecular biological studies of nucleic acids strongly declined in connection with the orientation of DNA research to well-defined oligonucleotides and viral or plasmid DNAs of known nucleotide sequences. The preparation of such DNA samples was expensive and laborious, requiring better highly sensitive analytical methods. In the middle of the 1980s we introduced the adsorptive stripping techniques in the DNA analysis increasing the sensitivity of the DNA determination by about 2 orders of magnitude.[22-24] At the same time we started our work with the DNA-modified electrodes,[23-25] and within less than 10 years the DNA-modified electrodes prevailed in the field (reviewed in Refs. 1-3, 5, 26).

In 1986 we showed that DNA and RNA can be easily immobilized at mercury and carbon electrodes by immersing the electrode in a small drop (3 to 10 µl) of a NA solution for a short time.[23,25,27] Due to strong adsorption of the nucleic acids a stable layer formed at the electrode surface; the electrode was then washed and the voltammetric measurements performed in solutions not containing any NA. This technique has been called adsorptive transfer stripping voltammetry (AdTSV). Later covalent immobilization of NAs at electrodes (e.g., binding of oligonucleotides end-labeled by sulfur at gold electrodes) has became popular in connection with development of the DNA hybridization sensors.[1-3]

C. Labeling of Nucleic Acids with Electroactive Markers

Reduction and oxidation of nucleic acids at electrodes is irreversible, producing signals at highly negative or positive potentials; no analytically useful catalytic signals have been observed with natural nucleic acids. At the beginning of the 1980s, the first electroactive markers were covalently attached to DNA.[28-31] Based on osmium tetroxide complexes with nitrogen ligands (Os,L), they formed stable DNA-Os,L adducts producing redox couples and yielding a catalytic signal at about –1.2 V at the mercury electrode.[31,32] Some Os,L complexes showed a useful selectivity for ssDNA. Electrochemical studies of the osmium tetroxide, 2,2'-bipyridine (Os,bipy) and other Os,L complexes led the way to a wide application of these compounds as probes of the DNA structure *in vitro* and *in vivo* in connection with biochemical methods, including DNA sequencing and immunoassays (reviewed in Refs 32-34). Most of the DNA-Os,L adducts can be determined electrochemically with a high sensitivity;[31,32,35,36] in AdTSV experiments

with 3 μl analyte volume, femtomoles, or hundreds of attomoles of ss 20-mer ODNs (depending on their base composition) can be detected.

III. DEVELOPMENT OF DNA DETECTORS AND SENSORS

A. DNA Damage

Damage of the genetic material causes serious disturbances in living processes. An accumulation of mutations and/or other kinds of DNA damage increases carcinogenic or teratogenic risks. Electrochemical methods can be used (a) for the detection of DNA strand breaks and base damage, and (b) for detection of electroactive substances that specifically interact with DNA (covalently and/or noncovalently). The significance of the electrochemical detectors or sensors for DNA damage is closely connected with their sensitivity. The detection of the damage to DNA bases has usually relied on the decrease of the signal of the damaged base (e.g., guanine), allowing the detection of one base among about 20 to 50; such a low sensitivity of the electrochemical methods can hardly compete with the available biochemical methods. Satisfactory sensitivity was obtained with the detectors for DNA strand breaks using a DNA-modified mercury electrodes (one DNA strand break among $>2 \times 10$ intact sugar-phosphate bonds).[37-40] The sensitivity of the detection of compounds binding covalently or noncovalently to DNA depends on the electrode process (particularly the yield of electrons) to which the given compound is subjected. The electrochemical detection of the DNA damage is a very promising field; the number of papers is increasing,[41-44] but many possibilities have not been yet exploited fully. The reader can find more details in the recent reviews.[3,38,41]

B. DNA Hybridization

1. Conventional Sensors with Single Stranded DNA Probes Immobilized at the Electrode

In DNA hybridization sensor, a short single-stranded oligodeoxynucleotide (ODN, DNA probe) is immobilized on an electrode to create the recognition layer. The DNA-modified electrode is immersed into a solution of target DNA in which the nucleotide sequence should be tested. If the sequence of target DNA matches that of the probe DNA (based on the complementarity principle stating that A pairs with T and G with C), a hybrid (probe-target) DNA duplex forms at the electrode surface.

The detection of the hybridization event is usually based on different properties of duplex vs. ssDNA, such as changes in conductivity or in adsorption/desorption signals or on signals of redox indicators binding preferentially to the duplex DNA (intercalators and minor groove binders) (Figure 1a). These methods are useful in model experiments with synthetic ODNs where probe and target DNAs are of about the same size. They are, however, less suitable for a real DNA sequence analysis of viral or chromosomal DNAs, or longer PCR products where the target DNAs is much longer than the probe. Alternatively presence of target DNA at the electrode surface (Figure 1b,c) can be detected by either measuring signals due the natural electroactivity of DNA (electrooxidation of guanine was utilized for this purpose [Figure 1c][1, 5, 45-47]) or by prelabeling target DNA molecules by an electroactive marker (Figure 1b); enzymes such as peroxidase or alkaline phosphatase (used frequently in immunoassays) followed by electrochemical measurement of changes in concentration of the substrate or product of the enzymatic reaction[48] have been used for this purpose. The ability of the electrochemical DNA hybridization detector to recognize a single-base mismatch (point mutation) was demonstrated for the first time in 1996 using peptide nucleic acid (PNA) as a probe. Since that time several other techniques of the point mutation detection have been developed.[3,49,50]

For highly specific and sensitive DNA hybridization analysis, it is critical that the nonspecific DNA adsorption must be negligibly small. Fulfilling this condition with the procedures shown in the above scheme (Figure 1) is very difficult. Interfacing the electrode by thioalkanes (at gold electrodes)[51] or by conducting polymers[48] yielded good results with relatively short DNA molecules. To our knowledge, so far no electrochemical

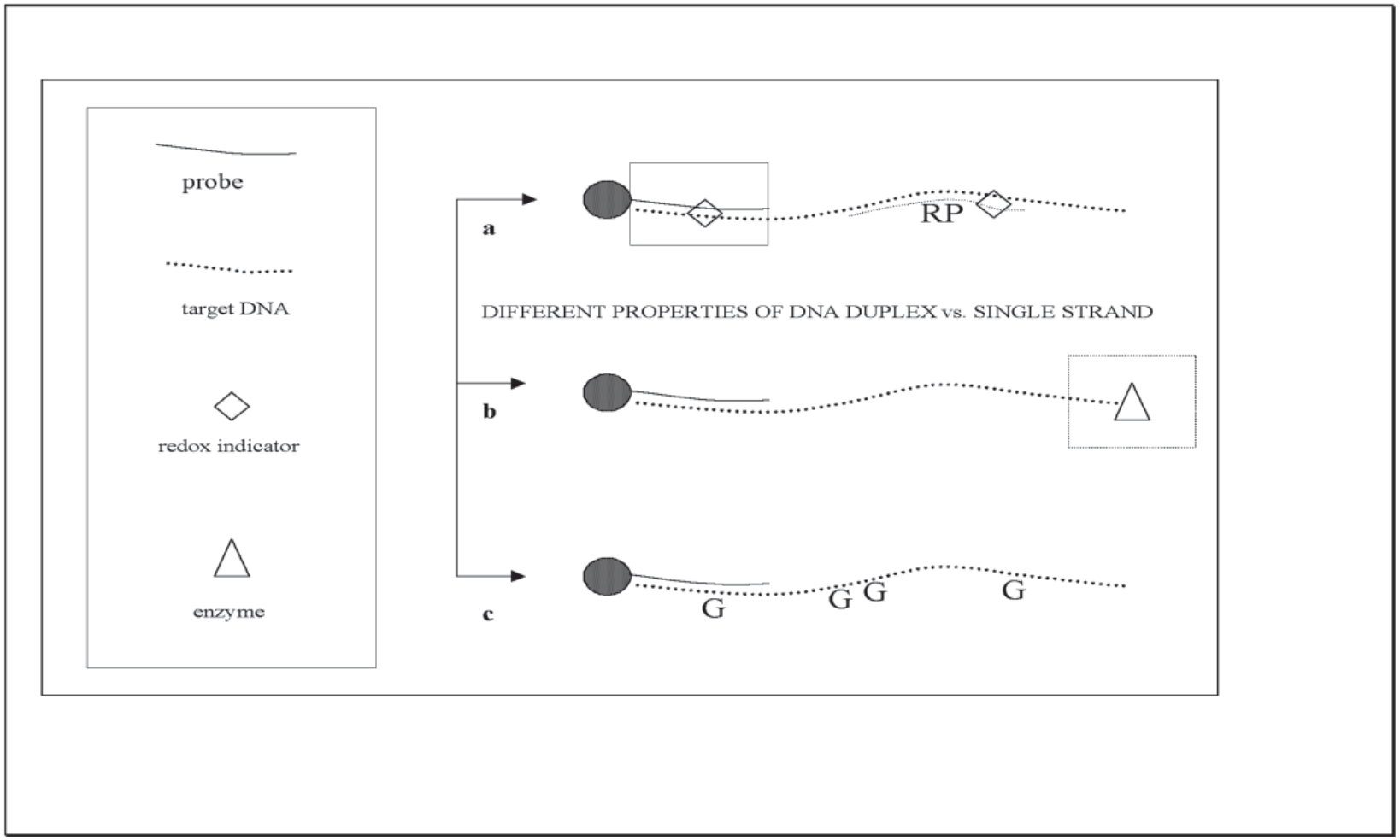

FIGURE 1. Scheme of DNA hybridization and electrochemical detection at a single (electrode) surface; various ways of detection. Probe DNA (usually a single stranded (ss) 15- to 20-mer oligodeoxynucleotide) is immobilized at the electrode surface and hybridized with a target DNA in solution. On interaction of the probe with the complementary target DNA, the DNA duplex is formed. **(a**) Formation of the duplex at the electrode surface is manifested either (1) by changes in the properties (conductivity, a.c. impedance, etc.) of the DNA duplex vs. ssDNA, or (2) by a redox indicator binding preferentially to the DNA duplex, (3) by a reporter probe (RP), which is end-labeled by an electroactive marker;[63] RP has to be complementary to a DNA sequence near to the duplex (located close to the electrode surface). The presence of target DNA is detected at the electrode surface **b**, with end-labeled DNA by means of the electroactive marker signal or **c**, in label-free detection using the signal due to oxidation of guanine residues.

method for the sequence determination in long DNAs such as chromosomal or viral ones has been published.

2. New Conceptions

a. Hybridization and Electrochemical Detection at Two Different Surfaces

To overcome difficulties with the analysis of longer DNA molecules, we recently have proposed to separate DNA hybridization from the electrochemical detection of the hybridization event. We hybridized DNA at one surface (surface H, with immobilized DNA probe) and performed the electrochemical detection at the detection electrode (DE, without immobilized probe) best suited for the electrode process (Figure 2A). In our first experiments we choose commercially available magnetic Dynabeads Oligo (dT_{25}) (DBT) originally developed for isolation of mRNA.[52] As the detection electrode we used either carbon or mercury electrodes[53-55] (Figure 2B). Due to strong hydrophobic interactions of the nucleic acid base with the surface of the mercury electrodes, these electrodes appear not well suited for the DNA hybridization.[55] On the other hand, mercury film and dental amalgam electrodes may well serve as DE. Examples of the use of these electrodes in DNA hybridization sensors are in Figure 3.

In addition to the highly sensitive detection of the DNA-Os,L adducts at mercury electrodes these adducts can be analyzed also at carbon and other solid electrodes using specific polyclonal or monoclonal antibodies[32,56] labeled with an enzyme (e.g., peroxidase, alkaline phosphatase, etc.) (Figure 4). In this case changes in concentration of the given substrate or product of the enzymatic reaction are determined. For example, we used the alkaline phosphatase-labeled antibody and 1-naphthyl phosphate as an electrochemically inactive substrate and measured the concentration of the oxidizable product (1-naphthol) at carbon electrodes.[54] In this way, we detected the Os,L-labeled DNA 97-mer and 67-mer oligodeoxynucleotides and a DNA PCR product (226 base pairs) captured selectively at DBT at high sensitivities. Electrochemical enzyme-mediated detection of the DNA hybridization event is not limited to the immunoassay of the DNA adducts; target DNA can be also enzyme labeled using, for example, the well known avidin (streptavidin)/biotin system. This approach has been used recently by Wang et al.[57] in combination with magnetic beads covalently coupled with streptavidin to detect a relatively short ODN (19 nucleotides). These authors also used streptavidin-coated gold nanoparticles to label the target DNA and determined the dissolved gold at carbon electrodes.[58]

b. Other

The application of double-stranded DNA as a probe and other possible approaches in the development of electrochemical sensors have been little utilized.[3,59]

IV. CONCLUSIONS

Recent progress in the development of the DNA hybridization sensors is encouraging. Performing DNA hybridization and the detection of the hybridization event at two different surfaces ("double surface" technology) offers a number of new possibilities. By choosing DBT as surface H new interesting properties (not offered by the electrodes used so far for DNA hybridization) became available. For example, using 100 μg of DBT (20 μL of the DBT suspension) up to 7 cm^2 area can be obtained,[60] which is much larger than the area of the electrodes usually used in hybridization detectors. DBT are easily transportable by means of pumping and magnetic field systems and can be easily incorporated in microfludic devices.[61] After a selective capture of target DNA at DBT the sample volume can be minimized and the amount of the target DNA determined with a small electrode (or a microelectrode) best suited for the given purpose. It appears that the "double surface" technologies will have a better chance to succeed in the analysis of long PCR products, viral, and chromosomal DNA fragments than the currently used single surface techniques.

It can be expected that the interest in electrochemistry of nucleic acids will further increase in

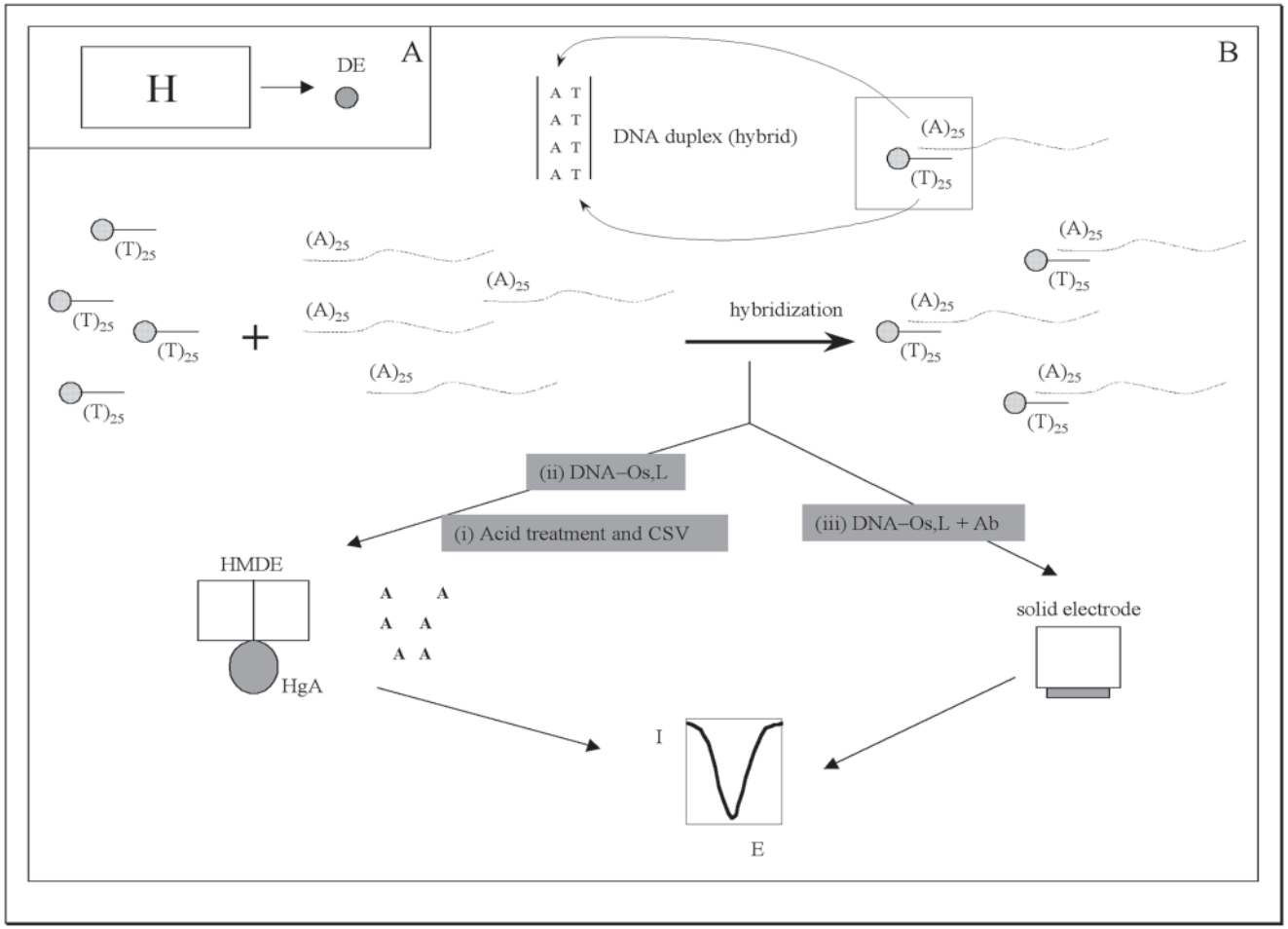

FIGURE 2. DNA hybridization and electrochemical detection of the hybridization event at two different surfaces. (A) DNA hybridization is performed at a relatively large surface best suited for this purpose (surface H), while a small detection electrode (DE) is used to monitor the hybridization. (B) The scheme of DNA (or RNA) hybridization in which DYNABEADS oligo$(dT)_{25}$ are used as surface H and mercury or solid electrodes as DE. The detection can be based either on (1) the cathodic stripping voltammetry (CSV) of adenine released from unlabeled DNA (or RNA) by acid treatment.[54] Adenine produces a sparingly soluble compound with the electrode mercury, or (2) modification of the DNA by osmium tetroxide complexes with nitrogen ligands (Os,L) and determination of the DNA-Os,L adduct at carbon[53] or mercury electrodes,[36] or (III) enzyme-linked immunoassay with electrochemical detection at solid electrodes.

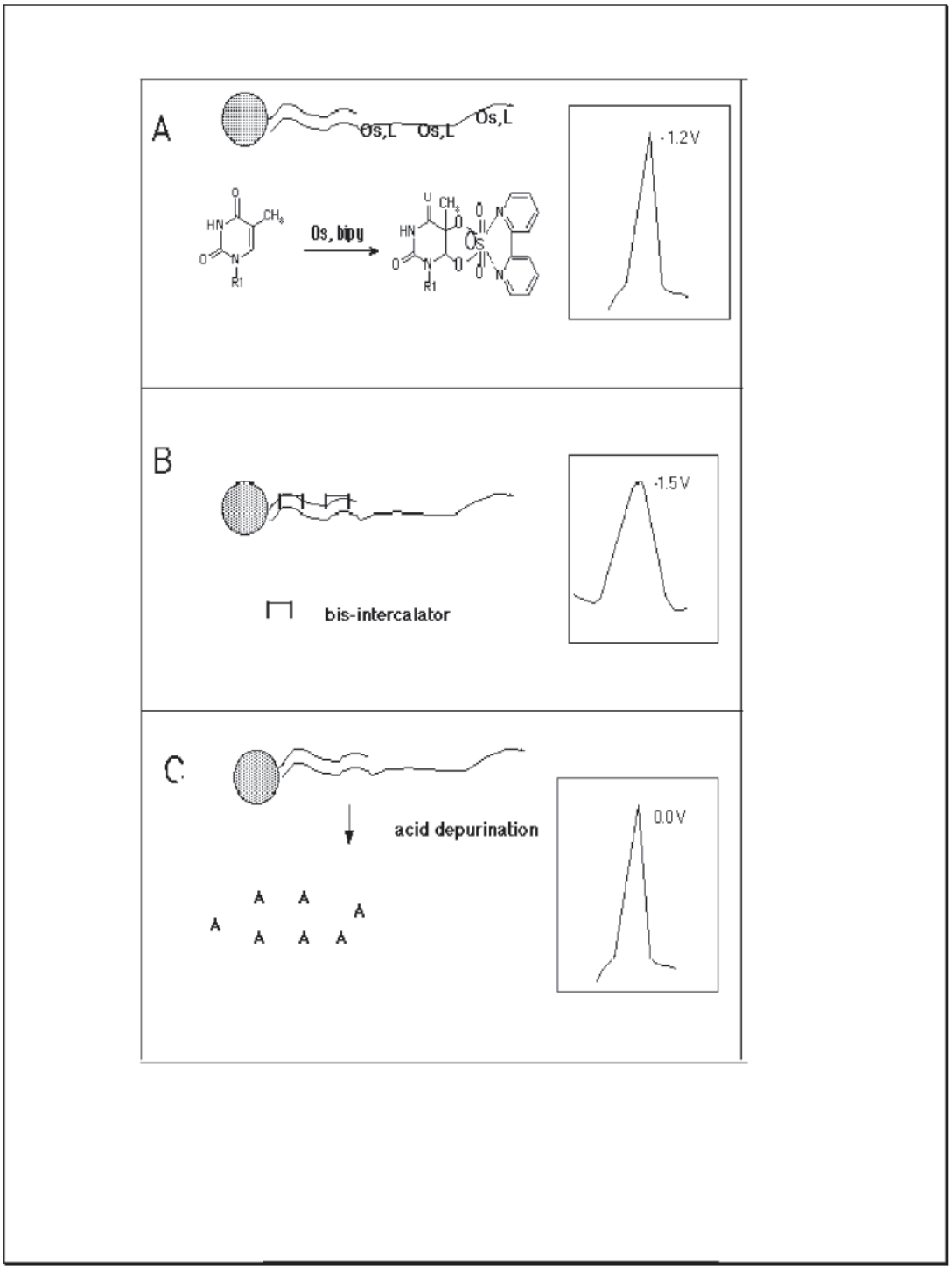

FIGURE 3. Examples of the detection of the hybridization event using mercury or dental amalgam as DE. **(A**) Target DNA is labeled with osmium tetroxide complex (Os,L) such Os, 2,2'-bipyridine binding to thymine residues in single-stranded DNA.[3,35] Voltammetric peak at about –1.2 V of the target Os,L-modified DNA, due to the catalytic hydrogen evolution, is measured at the mercury electrodes. Detection limit < 1 ng DNA/ml; pg amounts of the DNA are sufficient for the analysis. **(B**) The formation of the duplex DNA is detected by a redox indicator, which is a *bis*-intercalator.[64] Similarly to the threading intercalators,[65] the *bis*-intercalators bind duplex DNA more tightly than simple intercalators; thus they are better suited as redox indicators than simple intercalators and minor groove binders. Detection limit of the catalytically active *bis*-intercalator < 25 ng/ml.[66] **(C**) Label-free detection of target DNA based on the cathodic stripping voltammetry (CSV) of adenine released from DNA (or RNA) by acid treatment. Adenine produces a sparingly soluble compound with the electrode mercury. Detection limit: about 5 ng DNA/ml.[54]

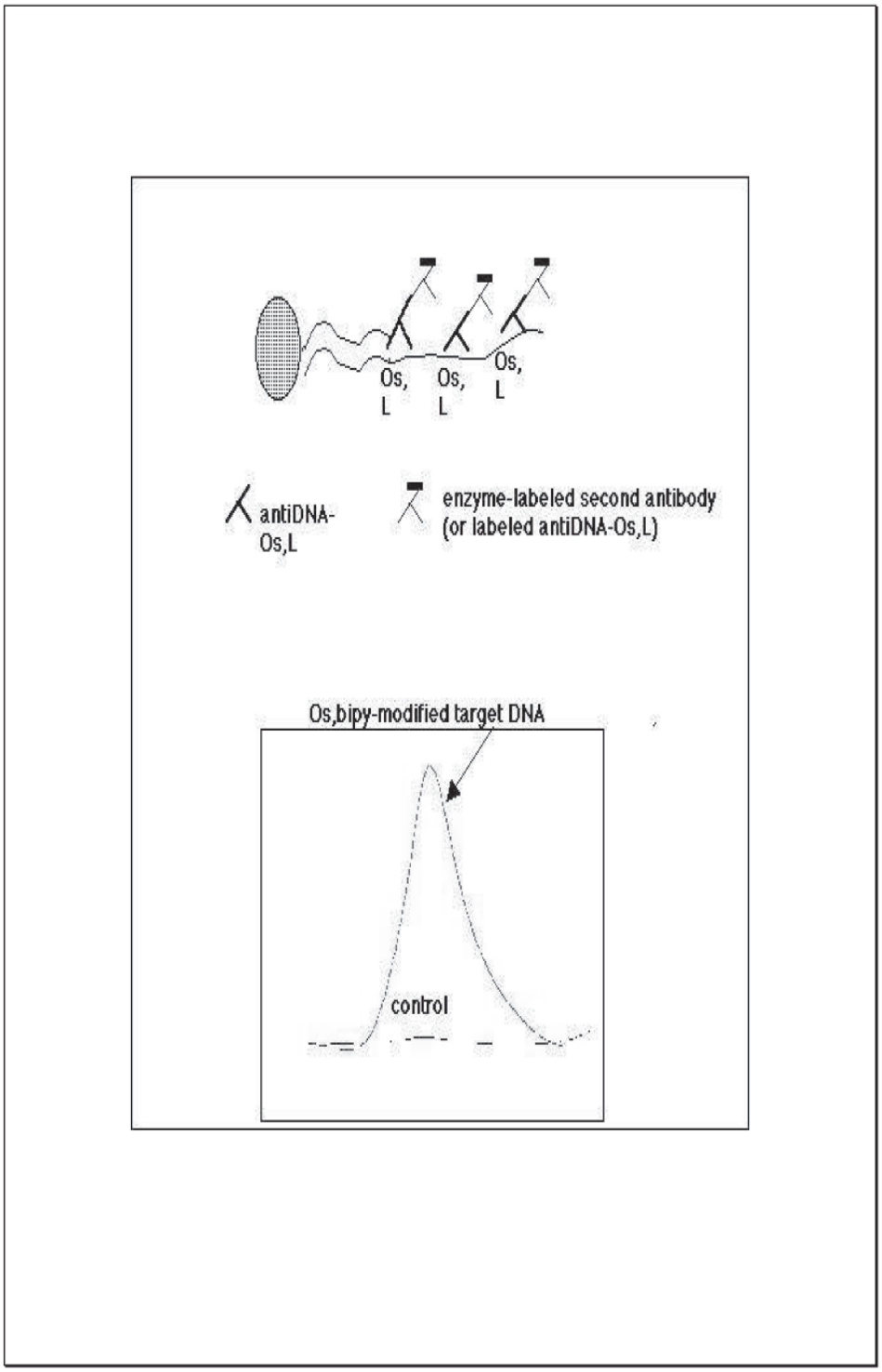

FIGURE 4. The use of enzyme-linked immunoassay in the detection of the DNA hybridization event. Target DNA is modified by osmium tetroxide, 2,2'-bipyridine (Os,bipy, see Figure 3A). DNA-Os,bipy adducts are immunogenic and can be detected by antibodies,[56] polyclonal or monoclonal antibodies against the DNA-Os,L adducts (anti-DNA-Os,L). The antibody can be labeled by an enzyme such as alkaline phosphatase or a second antibody labeled with an enzyme can be used. Using the alkaline phosphatase as an enzyme, the electroinactive 1-naphthyl phosphate was applied as a substrate and the electroactive product (1-naphthol) determined at carbon electrodes.[67] By means of this technique in combination with DBT (see Figure 2), relatively long DNA fragments were determined. Alternatively, target DNA can be end-labeled by enzymes using biochemical techniques, such as avidin (streptavidin)-biotin systems.[57]

the near future, and various types of electrochemical DNA hybridization devices will appear soon on the market. The present state of electrochemistry of nucleic acids was documented in a special issue of Talanta on *"Electrochemistry of nucleic acids and development of electrochemical DNA sensors"* which was published quite recently (April 2002). An encyclopedic survey of this topic will be published in 2002 (Ref. 62).

ACKNOWLEDGMENTS

This work was supported by grant No. 204/97/K084 from the Grant Agency of the Czech Republic, by grant No. Z 5004920, A 4004901 and S 500 4107 from the Academy of Sciences of the Czech Republic and by november AG, Erlangen, Germany.

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