CHEMICAL REVIEWS

Review

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Electrochemistry of Nucleic Acids

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CONTENTS

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1. INTRODUCTION AND SCOPE

The present boom in electrochemical studies of nucleic acids (NAs) is closely related to DNA sequencing techniques, which are indispensable in genomics. In the first half of the 1960s, it was believed that in difference to the RNA nucleotide sequencing,[1] sequencing of large genomic DNA would not be possible. Methods of DNA renaturation/hybridization were, however, available since 1960.[2,3] The capacity of DNA to form molecular hybrids was used to test the genetic relatedness of some organisms, to study the specificity of hybridization of DNA with mRNA[2−4] and for other purposes.[5] The discovery of sequence-specific restriction endonucleases by the end of the 1960s[6] opened the door to specific cleavage and manipulation of DNA.[7] In about a decade, Maxam and Gilbert[8] and Sanger et al.[9] invented the technology underlying DNA sequencing based on gel electrophoresis, that is, an intrinsically slow method. Shortly afterward, solid-supported DNA hybridization using membrane blotting was applied for DNA analysis.[10] This technology has become popular among biochemists and molecular biologists[11] but with the arrival of genomic sequencing,[7] other techniques suitable for automated parallel DNA analysis have been developed. In the early 1990s, array technologies based on the immobilization of multiple specific DNA fragments or ODNs onto solid surfaces and the detection of DNA duplexes resulting from hybridization with complementary target DNA (tDNA) appeared as promising tools for DNA sequencing. Such detection required fluorescence or radioactive labeling of DNAs. These array technologies have greatly influenced genomics and proteomics and further development in this field still continues, seeking faster, more sensitive and specific and/or label-free methods based on various principles, including electrochemical (EC) ones. We believe that NA electrochemistry can still offer a number of interesting approaches, which can be particularly useful in decentralized DNA analysis.

Electrochemistry of NAs fits in the field of Biomolecular Electrochemistry defined by J.-M. Saveant through a double motto: "molecules for electrochemistry and electrochemistry for molecules."[12] It is encouraging that unlike in the past,[13,14] at present a large number of electrochemists consider NAs as molecules for electrochemistry. In this article we wish to emphasize the latter part of this motto and show that electrochemistry is not only an excellent tool but also an interesting approach to studies of these prodigious biomacromolecules.

In this review, the present state of electrochemistry of NAs and its application in sensors for DNA hybridization and DNA damage will be briefly reviewed. In the following paragraphs, special attention will be paid to (a) relations between DNA structures and their EC responses, including EC molecular beacons utilized in DNA hybridization sensing and the effects of the electrode charge on the structure of surface-immobilized DNA and (b) the recent progress in the development of DNA hybridization sensors working with biologically relevant NA samples with or without amplification by polymerase chain reaction (PCR). The article also details that the knowledge of NA electrochemistry can be applied to solve various biochemical problems and to obtain new information about the properties and behavior of NAs at charged interfaces.

1.1. Electrochemistry of Nucleic Acids is a Booming Field

The interest of scientists in electrochemistry of NAs has increased dramatically in the recent two decades as documented by an increase in the number of scientific publications in this science area (Figure 1). Between 1960

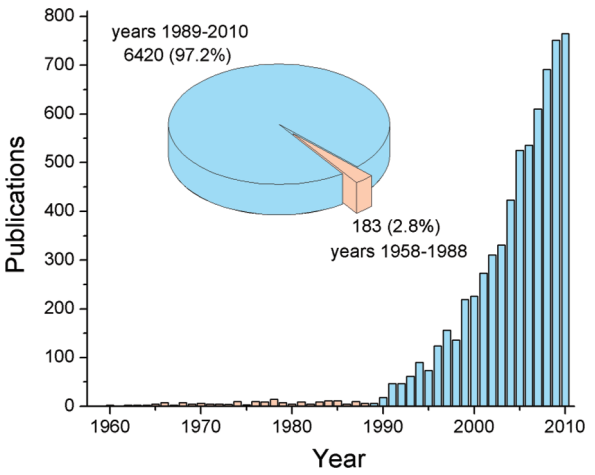

Figure 1. Papers on Electrochemistry of Nucleic Acids between 1958 and 2010. The graph is based on Web of Science query for (a) (polarograph* AND DNA) OR (electrochem* AND DNA) OR (electrochem* AND "nucleic acid*") in *Topic* and (b) in *Year Published*. In 1949−1957, only one paper was found; this paper claimed polarographic inactivity of nucleic acids. Between 1958 and 1987, the above numbers were corrected by excluding papers out of the field and by adding papers obtained through searching in *Author* for: Berg H., Miller I. R., Nurnberg H.W., Palecek E., and Reynaud J. [i.e., the scientists who (to our knowledge) significantly contributed to the field in the given period of time]. Starting from 1988, the publication numbers were taken from the Web of Science without any corrections. Adapted with permission from ref 13. Copyright 2009 Wiley-VCH.

and 1989, an average ∼10 papers were published per year in this field, while in 2010 alone, about 760 papers appeared. Within the past 50 years altogether over six thousand papers on NA electrochemistry have been published. In other words, in the first three decades of NA electrochemistry (let us call it the *Lag period*) only about 2.8% of the material was published in contrast to about 97.2% in the last 20 years (*Exponential period*); about 34% of this growth has occurred solely within the 2008−2010 time frame. Various questions can be asked, such as "What is the reason for this remarkable increase?", "How long will this exponential growth last?", "Is the amount of knowledge gained in the recent 20 years equal to >97% of what we know about the NA electrochemistry?", etc. We shall attempt to answer some of these questions in the following chapters.

1.2. Progress in Genomics Influences Electrochemistry of Nucleic Acids

Different reasons can be proposed for the appearance of the *Exponential period* but perhaps the main one lies in biology and

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particularly in the progress in genomics and in the Human Genome Project. For example, new tailor-made medicine anticipated in this century requires data on the differences in the genomes and in the gene expression of individuals. Such data dealing with DNA nucleotide sequences can be utilized in the medical treatment of individual patients. In the case of cancer, a majority of patients benefit from chemotherapy, while a significant amount of them may still resist the treatment with a specific anticancer drug. The time lost by treatment with an ineffective drug and its side effects may cause serious harm to the patient undergoing therapy. Recently, it has been found that a specific mutation in an oxidoreductase gene results in resistance to breast cancer chemotherapy with anthracycline drugs.[15] It can be expected that soon it will be possible to identify individuals who would benefit most from various treatment regimens. Screening the genomic DNA of individual patients for specific mutations will become an important step preceding the decision on the drug choice for the therapy of the given cancer patient.

Using classical methods for sequencing human genomes with 3×10^9 base pairs is difficult and too expensive.[7] DNA hybridization arrays with optical detection are currently applied in research laboratories and are gradually entering clinical medical laboratories in large hospitals. Electrochemistry offers a simpler and less expensive alternative to optical detection. Such detection can be particularly useful in delocalized, moderately parallel DNA analysis, for example, in small hospitals or in a doctor's office. The outlook for practical applications of the principles of DNA electrochemistry has resulted in a plethora of papers aimed at creating a simple, inexpensive and practical device for the detection of specific DNA sequences, favored by physicians and patients, in analogy to blood glucose meters at present.[16−18] EC detection of DNA damage, which may result in harmful mutations, represents another interesting challenge to DNA electrochemistry. Clearly, the enormous increase of interest in NA electrochemistry in the last two decades has been driven by the outlook for the practical application of EC DNA sensing in biosensors, arrays and chips for delocalized DNA sequencing and detection of DNA damage.

DNA sensing is usually based on the formation or disruption of the DNA double-helical structure and some specific properties of this biomacromolecule. The double-helical structure of DNA was proposed in 1953 by Watson and Crick on the grounds of Chargaff's analysis of the base content of a number of DNA samples from different organisms, as well as through X-ray analysis of DNA fibers.[19] X-ray crystallographic analysis of DNA at that time was not possible, mainly because of lack of synthetic oligodeoxynucleotides (ODNs), which were necessary for DNA crystallization. In spite of very limited structural information from the analysis of DNA fibers, Watson and Crick ingeniously invented the right structure of DNA. Several years later, it was shown that under certain nonphysiological conditions (extreme pH values, high temperatures, nonaqueous solutions, etc.), the DNA double helix can collapse and the DNA strands separate. This process was called DNA melting or denaturation and it has been described in detail.[3] Another important discovery was made in 1960 by J. Marmur and P. Doty (at the Harvard University). They showed that, under certain conditions, the denaturation can be reverted and the separated strands are capable to reform their double-helical structure. This process was called DNA renaturation or hybridization[2,3] and it has become an indispensable part of many molecular biotechnologies. In the same year it was shown that the EC signals of native double-stranded (ds) DNA and degraded single-stranded (ss) DNA greatly differ,[20] suggesting that electrochemistry may represent a new method for tracing DNA denaturation and renaturation. J. Marmur immediately recognized this opportunity and invited one of us (E.P.) to join his laboratory as a postdoc. The story of the 2-year delay in E.P.'s departure to the U.S.A., as well as the 9 months transportation of the EC device via air cargo from the former Czechoslovakia to the USA and breakage of this device on its route to its destination was briefly discussed.[21] We believe that, in that time, the newly born NA electrochemistry field had a unique chance to become a method of choice in the booming DNA research. This chance was lost, however, because of impermeability of the Iron Curtain for ideas and scientific instruments in the time of the Cold War. It took about 30 years before another chance for the boom of the NA electrochemistry arose.

1.3. Electrochemical DNA Hybridization Sensors

The ability of target ssDNA to form a duplex with the ss probe indicates that the nucleotide sequence of the tDNA is complementary to the sequence of the probe (Figure 2). In other words, formation of the duplex provides evidence that the target has the expected nucleotide sequence. EC detection of the hybridization event (formation of a DNA duplex) is based on the EC signals due to NA electroactivity, labeling of the target or the probe with electroactive (covalently bound) species, including nanoparticles, or changes in various electrochemically detectable DNA properties, which are related to

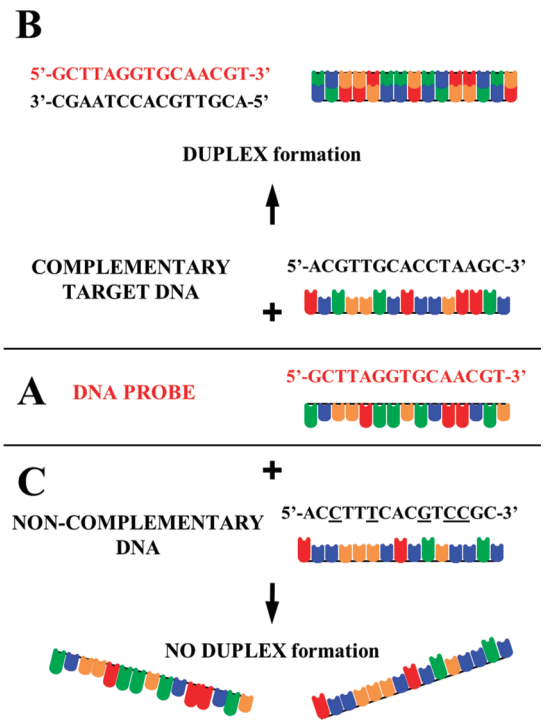

Figure 2. DNA hybridization scheme. (A) In EC DNA hybridization sensors/detectors, probe DNA is usually immobilized at electrode or other surfaces. (B) Scheme of DNA duplex formation from two oligodeoxynucleotide (ODN) strands - DNA probe (red) and complementary target (black). Formation of the DNA duplex (indicating complementarity of the two DNA strands) is detected electrochemically. (C) Noncomplementary ODN does not form duplex with the DNA probe.

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changes in the DNA structure resulting from the hybridization step. The application of redox indicators that bind preferentially to dsDNA (such as intercalators and groove binders), which was popular in the second half of the 1990s, now appears as a less attractive possibility because these indicators also bind to ssDNA, thus increasing the background response. On the other hand, it cannot be excluded that studies of the compounds that bind very tightly to dsDNA, such as bis-intercalators[22,23] or threading intercalators,[24] and specific enhancement of their signals[25] will produce some useful results in special cases of NA electrochemistry research.

Good knowledge of NA structure and properties is an important prerequisite for performing research into the electrochemistry of NAs and the development of the EC DNA sensors. DNA structure and properties in connection with electrochemistry have been recently summarized in an excellent review by Compton et al.[26] and also in some book reviews.[27,28] Some problems of NA structures and properties related to their EC analysis will be discussed in section 3.2.3.

Considering the large number of reviews, which are more or less focused on specific research areas (Table 1), we believe it would be unreasonable to write a comprehensive review article on EC DNA hybridization. Instead, in the following sections we only briefly summarize the first 30 years of EC DNA research (section 2), followed by a summary of the electroactivity of NAs and NA labeling (section 3). Section 4 focuses primarily on potential-driven changes in the conformation of surface-attached DNA, while section 5 deals with major advances in the development of DNA hybridization sensors. In the last sections, recent trends in the analysis of biologically relevant DNA and RNA samples (section 6), detection of DNA damage (section 7) and lab-on-a-chip/DNA arrays (section 8) are reviewed. The literature is covered since 2007, but when specialized reviews are available, the topic is only briefly summarized and the pertinent reviews are quoted. In section 4, dealing with the effect of potential on DNA structure, the topic (which has not been yet covered by a journal review) is covered from the beginning. There is no doubt that electrochemistry of NAs is now a booming field (Figure 1). But how did it begin?

2. EARLY STUDIES AND THE LAG PERIOD

The first papers on the electrochemistry of NAs were published about 50 years ago.[20,96,97] For the first two decades, all data on NA electrochemistry was obtained with mercury electrodes. By the end of the 1950s, it was difficult to imagine that the EC analysis of DNA and RNA could start with any other electrode because, at that time, DC polarography with the dropping mercury electrode (DME) dominated the EC research.

Soon it was discovered that polarographic responses of native dsDNA differ from that of denatured or degraded ssDNAs[20,98−103] and polarographic methods can be used to study DNA denaturation, renaturation, and premelting.[104] The beginning of NA electrochemistry was closely related to the so-called oscillographic polarography with controlled AC (OP, cyclic AC chronopotentiometry, or cyclic reciprocal derivative chronopotentiometry[105] according to the present nomenclature) invented by J. Heyrovsky in 1941.[106,107] OP worked with DME, but in contrast to DC polarography (requiring a number of mercury drops dripping from the capillary during the recording of a single polarogram) (Figure 3A), OP enabled the whole analysis to be performed in seconds at a single mercury drop (Figure 3B). Predominantly derivative curves were recorded, such as dE/dt against E. This method displayed a cathodic signal (indentation) CI-2 because of the reduction of cytosine (C) and adenine (A), which was specific for ssDNA (Figure 3B) and a less negative capacitive CI-1, produced by both ds and ssDNA. In addition, these DNA forms as well as RNA yielded an anodic signal AI (at ∼−0.3 V), which was due to oxidation of the guanine (G) reduction product formed at highly negative potentials (∼−1.8 V).[20,97,102,103,108−111] In denatured ssDNA this indentation was always much deeper than the signal in the parent dsDNA under the same conditions. In contrast to the opinions of most of E.P.'s colleagues, electrochemists, who believed at the time that DC polarography was better suited for DNA analysis than OP, the former method was poorly suited for this purpose; it was much slower, required high DNA concentrations and showed other drawbacks,[13,112] making DC polarography not competitive with UV absorbance measurements commonly used in DNA analysis in the 1960s. In 1966, Barker's differential and normal pulse polarography (DPP and NPP, respectively), offering better sensitivity and resolution than OP indentations CI-1 and CI-2, were applied in studies of NAs (Figure 4B).[99] Naturally, experiments were done exclusively with long chromosomal DNAs from eukaryotic and prokaryotic sources as well as with viral DNAs because of lack of synthetic ODNs at that time. On the other hand, biosynthetic polynucleotides with an usual average molecular weight (MW) of 10^5 to 10^6 Da, and DNA degradation products, such as apurinic or apyrimidinic acids, were applied early to clarify the role of individual base residues in the electrode processes.[20,103,104,113,114]

Adsorption behavior of NAs was studied for the first time by I.R. Miller, who measured the differential capacitance of the DME immersed in solutions of DNA and RNA at pH 6 and showed that these NAs were adsorbed in the potential range from about 0 to −1.1 V, followed by desorption at ∼−1.2 V.[115,116] Further work showed that ssDNA may desorb at more negative potentials.[100,117,118] The ability of NA bases to associate at electrode surfaces was shown for the first time by Vetterl in 1966[119] and was later thoroughly studied.[120−122]

To our knowledge, in the 1960s it was only at the laboratory of one of us (E.P.) that EC reduction and oxidation processes of NAs was reported.[104,123] In the 1970s, H. W. Nürnberg (Jülich, Germany) and H. Berg (Jena, Germany) became interested in the electroreduction of DNA at mercury electrodes and gradually other laboratories in France (J. A. Reynaud), Poland (B. Czochralska and M. Wrona), England (G. C. Barker), Denmark (S. Kwee), and the U.S.A. (G. Dryhurst) became involved in NA electrochemistry.[124] For about 30 years, the electrochemistry of NAs was a small field involving only a handful of laboratories. Nevertheless, during this time a number of results was obtained (Table 2), some of which are now utilized in the research and development of DNA sensors. For example, the first DNA-modified electrodes,[125] electroactive labels covalently bound to DNA,[126−130] detection of the collapse and formation of the DNA double-helical structure[100−102] and potential-driven DNA unwinding at the electrode surface,[131,132] as well as the detection of DNA damage,[133] can be mentioned. More details on history of the NA electrochemistry can be found in recent reviews.[13,124]

2.1. From History to Present Time

It is interesting that some parts of the history of NA electrochemistry are now considered as granted, for example, oxidizability and reducibility of base residues in NAs, while others are ignored in spite of their importance for the present

Table 1. List of Journal Reviews Involving Various Aspects of NA Electrochemistry, Especially NA-Based Biosensors, Published in 2007−2011.[a]

first author (year)	title	ref
	intrinsic electroactivity and labeling of NA	
Fojta (2008)	Electrochemical stripping techniques in analysis of nucleic acids and their constituents	29
Hocek (2008)	Cross-coupling reactions of nucleoside triphosphates followed by polymerase incorporation. Construction and applications of base-functionalized nucleic acids	30
Hocek (2011)	Nucleobase modification as redox DNA labeling for electrochemical detection	31
Palecek (2009)	Fifty years of nucleic acid electrochemistry	13
	DNA charge transfer	
Boussicault (2008)	Electron transfer in DNA and in DNA-related biological processes. Electrochemical insights	32
Genereux (2010)	Mechanisms for DNA charge transport	33
Gorodetsky (2008)	DNA-mediated electrochemistry	34
Hill (2008)	Electrochemistry at the DNA/electrode interface: New approaches to nucleic acids biosensing	35
Merino (2008)	Biological contexts for DNA charge transport chemistry	36
	impedimetric sensors	
Bonanni (2010)	Use of nanomaterials for impedimetric DNA sensors: A review	37
Daniels (2007)	Label-free impedance biosensors: Opportunities and challenges	38
Lisdat (2008)	The use of electrochemical impedance spectroscopy for biosensing	39
Park (2009)	DNA hybridization sensors based on electrochemical impedance spectroscopy as a detection tool	40
	DNA hybridization sensors, in general	
Batchelor-McAuley (2009)	The physicochemical aspects of DNA sensing using electrochemical methods	26
Cagnin (2009)	Overview of electrochemical DNA biosensors: New approaches to detect the expression of life	41
Hvastkovs (2010)	Recent advances in electrochemical DNA hybridization sensors	42
Li (2008)	Recent development of interaction of transition metal complexes with DNA based on biosensor and its applications	43
Lucarelli (2008)	Electrochemical and piezoelectric DNA biosensors for hybridization detection	44
Luo (2009)	Electrochemical techniques on sequence-specific PCR amplicon detection for point-of-care applications	45
Palchetti (2008)	Nucleic acid biosensors for environmental pollution monitoring	46
Pedrero (2011)	Electrochemical genosensors based on PCR strategies for microorganisms detection and quantification	47
Pohanka (2008)	Electrochemical biosensors - principles and applications	48
Sadik (2009)	Status of biomolecular recognition using electrochemical techniques	49
Sassolas (2008)	DNA biosensors and microarrays	50
Simkova (2011)	Electrochemical DNA Biosensors and Flow-Through Analysis. A Review	51
Teles (2008)	Trends in DNA biosensors	52
Tosar (2010)	Electrochemical DNA hybridization sensors applied to real and complex biological samples	53
Wang (2008)	Electrochemical sensors for clinic analysis	54
	nanotechnology in DNA biosensors	
Abu Salah (2010)	Nanomaterials as analytical tools for genosensors	55
Carrara (2010)	Nanobiotechnology and sensing chips: New system for detection in personalized therapies and cell biology	56
Castaneda (2007)	Electrochemical sensing of DNA using gold nanoparticles	57
Choi (2010)	Nanotechnology for early cancer detection	58
Erdem (2007)	Nanomaterial-based electrochemical DNA sensing strategies	59
Kerman (2008)	Nanomaterial-based electrochemical biosensors for medical applications	60
	nanotechnology in DNA biosensors	
Lord (2009)	Nanomaterials for ultrasensitive electrochemical nucleic acids biosensing	61
Pandey (2008)	Prospects of nanomaterials in biosensors	62
Pingarron (2008)	Gold nanoparticle-based electrochemical biosensors	63
Pumera (2007)	Electrochemical nanobiosensors	64
Wang (2009)	Electrochemical sensors based on metal and semiconductor nanoparticles	65
Wang (2009)	Biomolecule-functionalized nanowires: From nanosensors to nanocarriers	66
Wang (2009)	Functional DNA directed assembly of nanomaterials for biosensing	67
Wei (2009)	Electrochemical biosensors at the nanoscale	68
Wei (2010)	DNA diagnostics: Nanotechnology-enhanced electrochemical detection of nucleic acids	69
Xu (2009)	Recent development of nanomaterials used in DNA biosensors	70
	use of carbon materials	
Agui (2008)	Role of carbon nanotubes in electroanalytical chemistry—A review	71
Ahammad (2009)	Electrochemical sensors based on carbon nanotubes	72
Daniel (2007)	A review of DNA functionalized/grafted carbon nanotubes and their characterization	73
Jacobs (2010)	Review: Carbon nanotube-based electrochemical sensors for biomolecules	74
Kim (2007)	Carbon nanotubes for electronic and electrochemical detection of biomolecules	75
Pumera (2010)	Graphene for electrochemical sensing and biosensing	76
Qureshi (2009)	Review on carbon-derived, solid-state, micro and nanosensors for electrochemical sensing applications	77
Rivas (2007)	Carbon nanotubes for electrochemical biosensing	78
Sanchez-Pomales (2009)	DNA-functionalized carbon nanotubes for biosensing applications	79
Shao (2010)	Graphene-based electrochemical sensors and biosensors	80
Vermeeren (2009)	DNA sensors with diamond as a promising alternative transducer material	81
Wang (2008)	Functionalized carbon nanotubes and nanofibers for biosensing applications	82
Yang (2007)	Carbon nanotubes for biological and biomedical applications	83
	sensors based on DNA structural changes	
Li (2010)	Target-responsive structural switching for nucleic acid-based sensors	84
Lubin (2010)	Folding-based electrochemical biosensors: The case for responsive nucleic acid architectures	85
Miranda-Castro (2009)	Structured nucleic acid probes for electrochemical devices	86
	RNA detection	
Cissell (2009)	Trends in microRNA detection	87
Hunt (2009)	Direct detection and quantification of microRNAs	88
	DNA chips and microarrays	
Arata (2008)	Toward single biomolecule handling and characterization by MEMS	89
Chen (2007)	Total nucleic acid analysis integrated on microfluidic devices	90
Choi (2011)	Microfluidic-based biosensors toward point-of-care detection of nucleic acids and proteins	91
Liu (2010)	Biofabrication to build the biology-device interface	92
Luong (2008)	Biosensor technology: Technology push versus market pull	93
Mir (2009)	Integrated electrochemical DNA biosensors for lab-on-a-chip devices	94
Mir (2011)	Electrokinetic techniques applied to electrochemical DNA biosensors	95

[a]Over 60 reviews were published between 2007 and 2010. Number of reviews published in 2011 is still growing.

research into the electrochemistry of NAs. Among the latter results, the unwinding of DNA immobilized at negatively charged electrode surfaces (including the effects of DNA topology on such DNA unwinding) can be mentioned. In the

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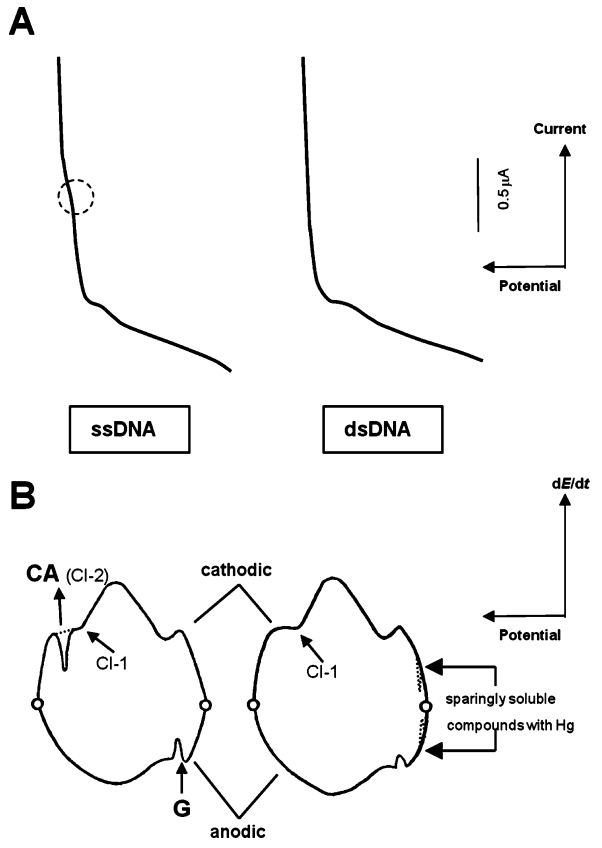

Figure 3. Comparison of DC polarography and oscillographic polarography of DNA. (A) DC polarograms of native (ds) and denatured (ss) calf thymus DNA at a concentration of 500 μg/mL, showing inactivity of dsDNA and poorly developed polarographic wave of ssDNA at about −1.4 V (dashed circle). (B) Oscillopolarographic curves dE/dt vs E. The upper part represented cathodic polarization in the range from about 0 to about −2 V, the lower part displays anodic polarization from −2 V back to zero. Presence of an electroactive substance (depolarizer) in the solution was manifested by indentations (incisions). Potentials of these indentations corresponded to half-wave potentials in DC polarography. Cathodic indentation CA (or CI-2, due to reduction of A and/or C) was characteristic for denatured ssDNA, while capacitive CI-1 was produced by both ss and native dsDNA. Both ss and dsDNA produced anodic peak G (due to oxidation of G reduction product), but the peak produced by dsDNA was smaller. (A) Reprinted with permission from ref 112. Copyright 1968 John Wiley and Sons. (B) Reprinted with permission from ref 21. Copyright 2002 Elsevier.

following paragraphs, we wish to briefly summarize the research into the relations between the EC responses of NAs on one hand, and NA structures on the other. More attention will be given to the problems of secondary changes in DNA structure occurring at the electrode interface.

3. REDUCTION, OXIDATION, AND ADSORPTION OF NUCLEIC ACIDS AT ELECTRODES

3.1. Electroactivity of Nucleic Acids Components

NA components, bases, nucleosides, and nucleotides, are electroactive species. Mercury electrodes are suitable for studies involving NA base reduction, while solid electrodes, especially carbon, are used for oxidation processes. Here, we shall limit ourselves only to brief summarization of the current knowledge, more detailed reviews can be found elsewhere.[13,28,32,104,118,120,143,144]

3.1.1. Reduction Signals. Polarographic reduction of usual NA bases was observed at DME around the middle of the 20th century, with adenine being the first base to be shown as electroactive.[145] Further studies revealed that A and C, along with their nucleosides and nucleotides, were reduced in aqueous media at acidic pH, with half-wave potentials ($E_{1/2}$) being −1.33 V for A and −1.44 V for C (vs SCE) at pH 4.2.[146−149] Reduction potentials for cytidine and cytosine nucleotides were slightly more positive than those of a parent base. $E_{1/2}$ of C and its derivatives shifted to negative values with increasing pH, suggesting an important role of protonation.[143] Reduction of A occurred at more positive potential than C and its reduction involved transfer of 4e$^-$ (as compared to 3e$^-$ process for C). Moreover, polarographic behavior of A was not appreciably changed when free or incorporated into nucleoside or nucleotides. When reaching potentials close to the electrolyte discharge, G became reduced, forming unstable, oxidizable product. Oxidation of G reduction product could be observed using e.g., cyclic or anodic stripping voltammetry, the latter consisting of the application of negative potential (around −1.8 V in neutral medium, usually of short duration), followed by potential scan in anodic direction.[111] Reduction of uracil (U) and thymine (T) was not observed in aqueous solutions, at least within the potential window of mercury electrodes. To unveil U and T reduction signals, usually complicated by the reduction of protons (from the water molecules), nonaqueous solvents with wider potential windows were employed. Using dimethylsulfoxide or acetonitrile, it was shown that both T and U underwent complicated reduction processes at highly negative potentials involving several radicals.[150,151] Recently, ionic liquids in combination with platinum electrodes were applied to study G reduction, which occurred at ∼ −2.2 V vs Ag.[152]

3.1.2. Oxidation Signals. Carbon electrodes are probably the most frequently employed electrodes in studies of oxidation of NA bases and their nucleotides or nucleosides. Purine bases require lower overpotential for oxidation than pyrimidines, with G being the most easily oxidized. Both A and G produce oxidation peaks in a wide pH range (0−12.5).[153,154] It was shown that A is oxidized in a process involving a total of 6 electrons per single A molecule to yield dicarbonium ion intermediate. The intermediate product is unstable and undergoes series of further reactions.[154] Oxidation of A was utilized in several recent papers on A detection.[155,156] G is electrochemically oxidized in 4 electron process to give an unstable intermediate, followed by further reactions yielding either parabanic acid or oxalylguanine.[153] Although purine bases were focus of most studies, T and C were also shown to produce oxidation signals.[157−161] Simultaneous determination of all four DNA monophosphate nucleosides using differential pulse voltammetry (DPV) at glassy carbon electrode (GCE), with a limit of detection being less than 1 μM for each nucleotide, was reported.[158] Similar results were obtained also at carbon paste electrode (CPE), using square wave voltammetry (SWV). Peak potentials of individual nucleoside monophosphates at neutral pH were +1.00 V (GMP), +1.28 V (AMP), +1.47 V (TMP), and +1.53 V (CMP) (vs Ag/AgCl), being rather positive.[159]

Oxidation signals of all four nucleotides were demonstrated also at graphene-based electrodes[160] and at nanocarbon films formed by electron cyclotron resonance sputtering method.[161] As compared to GCEs, both graphene and nanocarbon film provided oxidation signals of all four nucleotides also when present in short ODNs. Using graphite-epoxy composites with

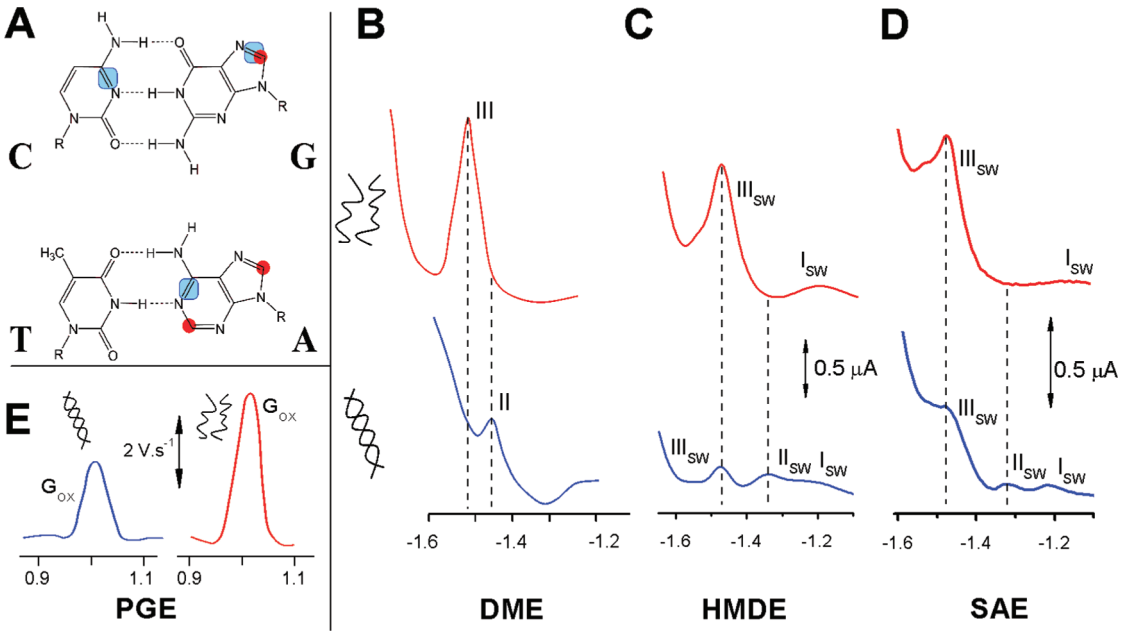

Figure 4. (A) Scheme of Watson–Crick base pairs and electroactive groups. Squares denote primary reduction sites at mercury electrodes, circles show primary oxidation sites at carbon electrodes. (B–E) Comparison of different methods and electrodes for the analysis of native (dsDNA, blue) and denatured DNA (ssDNA, red). (B) Differential pulse polarography (DPP) at dropping mercury electrode (DME) of 470 μg/mL dsDNA and 50 μg/mL ssDNAs. Note the absence of peak III in dsDNA and large difference in concentrations of ds- and ssDNA. (C–D) Square wave stripping voltammogram (SWV) of 20 μg/mL dsDNA and ssDNA at (C) hanging mercury drop electrode (HMDE) and (D) solid amalgam electrode (SAE), accumulation time, t_A, 60 s. (E) Oxidation peak of guanine (G_{OX}, baseline corrected) of 100 μg/mL dsDNA and 50 μg/mL ssDNA at pyrolytic graphite electrode (PGE) measured with constant-current chronopotentiometric stripping (CPS) analysis with baseline correction. (B) Adapted with permission from ref 123. Copyright 1971 Academic Press. (C–D) Adapted with permission from ref 193. Copyright 2011 Wiley–VCH. (E) Adapted with permission from ref 187. Copyright 2001 American Chemical Society.

Table 2. Important Findings Made in the First Three Decades (1958–1988) of the NA Electrochemistry

year	finding	ref
1958	DNA, RNA, and free bases are electroactive	97, 134
1960–1961	EC signals of DNA assigned to individual bases. Application of OP for probing the DNA structure	20, 103
1961	DNA adsorption at Hg electrodes	115, 116
1962–1966	EC studies of DNA premelting, denaturation and hybridization; tracing of ssDNA in dsDNA samples; effect of nucleotide sequence on dsDNA signals	99–102, 109
1966	application of DPP to ss and dsDNA analysis	99
1967	DNA damage detection	133
1967	DNA interaction with low MW compounds	135–137
1974	DNA surface unwinding	131, 132
1976	polymorphy of the dsDNA structure	138
1978	introduction of solid electrodes in NA analysis	139
1980	detection of NA bases with cathodic stripping voltammetry at nanomolar level	140, 141
1981–1983	Introduction of covalently bound electroactive labels to DNA	127, 128
1986–1988	Adsorptive transfer stripping voltammetry/ DNA-modified electrodes	125, 142

uneven surface, lower overpotentials for oxidation of DNA bases (than above-mentioned surfaces) were obtained. Free G base was oxidized at +0.35 V and free A at +0.63 V (vs Ag/AgCl); C and inosine showed, however, no peak.[162] Roughening of the surface led to more sensitive determination of DNA (mainly purine) bases, as shown with surface-roughened GCE[163] or mechanically grinded edge-plane pyrolytic graphite electrode.[164] Sensitive oxidation signals of G and A bases were also obtained by using cyclodextrin-modified poly(*N*-acetylaniline) at CPE.[165]

Because of a broad potential window of boron-doped diamond (BDD) electrode in aqueous media, direct oxidation of guanosine and adenosine could be observed.[166] This required an activation of the electrode by applying highly oxidizing potentials. When A and G were contained in a short ODN, oxidation peaks of A and G were only poorly developed due to the adsorbed G oxidation product. Recently, BDD in connection with liquid chromatography was used to simultaneously detect A, G, C, T and 5-methylcytosine (mC) in a single mixture, using amperometry as a detection method.[167] However, since a single potential value of +1.6 V (vs Ag/AgCl) was chosen for amperometry, lower peak currents from oxidation of C and T (as compared to G and A) close to the background discharge, were obtained. Anodically oxidized BDD was also used to study G and A oxidation signals of acid-hydrolyzed ODNs in presence of copper ions.[168] In this way, purine content of DNA could be estimated.

In addition to carbon materials, several authors have endeavored to apply other types of solid electrodes. Interestingly, A and C nucleotides could be traced down to submicromolar concentrations with copper electrodes due to the presence of a sugar which is electroactive at the copper surface.[169] Heller's group has recently examined behavior of guanine, guanosine, and guanosine monophosphate at redox polymer film-modified indium tin oxide (ITO) electrodes.[170] The proposed method was based on monitoring a catalytic oxidation of G by different redox polymer-coated electrodes, with the oxidation potentials of +0.81 V for G and +1.02 V for guanosine/GMP (vs NHE). EC oxidation of G residues at

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polycrystalline gold electrodes was also reported, however it has not been further exploited.[171]

3.1.3. Stripping Techniques. To increase sensitivity of detection, hanging mercury drop electrode (HMDE) in connection with cathodic stripping voltammetry (CSV) has been employed, enabling determination of NA components down to 10^{-9} M. CSV relied on formation of sparingly soluble compounds of NA purine nucleosides with the electrode mercury at positive potentials, followed by potential scanning in negative direction, thus reducing (or stripping) the previously generated mercury compounds. Pyrimidine nucleosides do not form sparingly soluble compounds with mercury because of an attachment of sugar to N1 position. Pseudouridine, in which the ribose residue is bound to C5, represents an interesting exception.[172]

Another option to amplify an intrinsic signal from DNA bases relies on a formation of sparingly soluble complexes of copper ions with A and G, leading to a sensitive determination of NA bases at mercury-based[173−177] and carbon-based[178,179] electrodes. This method was also employed in DNA hybridization sensors.[175,180] After hybridization of unlabeled tDNA with magnetic bead-immobilized capture probe, tDNA was hydrolyzed and released purine bases were detected either voltammetrically at HMDE[175] or potentiometrically at carbon electrode.[180] More about stripping techniques employed in NA analysis can be found in section 3.2.4.

3.1.4. Unusual NA Components. Besides bases and nucleosides frequently occurring in NAs, several unusual NA components were analyzed.[172,181−186] These included, for example, 5-fluorouracil (used in cancer treatment), methylated A (serving as a cell's protective system), pseudouridine (occurring in RNA, serving as a cancer marker), azidothymidine (antiretroviral drug for HIV), 7-deazapurines (capable of PCR incorporation, but with lower oxidation potentials), or methylcytosine (discussed in section 5.8). For example, 7-deazaguanine and 7-deazaadenine (in both the N7 atom is substituted with −CH group) were used for simultaneous oxidation of two DNA sequences on single ITO electrode, utilizing catalytic properties of metal mediators.[185] Two mediators were used in such a way that first mediator selectively oxidized 7-deazaguanine (having less positive oxidation potential) and the second mediator oxidized both 7-deazaguanine and 7-deazaadenine (being oxidized at a higher potential). Recently, direct oxidation of these derivatives, enzymatically incorporated into DNA, was reported.[186] Both derivatives produced oxidation signals at carbon electrodes at less positive potentials than their natural purine counterparts, although electrooxidation of 7-deazaadenine overlapped with an oxidation of G (peak G_{OX}), rendering it of little use in analysis. On the other hand, oxidation of 7-deazaguanine did not interfere with oxidation of natural purine bases, and may thus prove useful for estimation of DNA G + C content, the length of the amplified DNA fragments, or for tail-labeling of DNA probes for hybridization assays.

3.2. Electroactivity of Nucleic Acids

In this chapter, we wish to discuss faradaic processes occurring upon interaction of NAs with various electrode surfaces. Redox processes of NAs mostly involve reduction and oxidation of bases. NA reduction was observed only at mercury electrodes, while the oxidation was analyzed at different surfaces, including carbon, platinum, gold, silver, etc. The topic was thoroughly reviewed.[13,27,28,118,187−190]

3.2.1. Reduction Signals. Similarly to the behavior of free NA components described in section 3.1, A and C residues are reducible at mercury electrodes in single-stranded nucleic acids of various lengths (from short ODNs to very long chromosomal DNA, Figure 4A). Although U and T were found to be inactive in aqueous media, reduction of U in poly(U) chain was observed in nonaqueous solutions.[191] T reduction in DNA has not yet been observed. G is reduced at highly negative potentials (around −1.8 V at neutral pH), but it is more advantageous to study oxidation of its reduction product, as described in section 3.2.2.

Interestingly, A residues in NAs were reducible also at neutral pH (as compared to free A reducible only at acidic pH) if proper electrolyte, containing ions efficiently screening negative charges of phosphate groups, was used.[114] In absence of counterion atmosphere made of cations, polyanionic DNA would be strongly repulsed at neutral pH from the negatively charged electrode. For efficient screening, salts with large cations, for example, CsCl or ammonium formate have been commonly used.[104,114,192]

3.2.2. Oxidation Signals. Currently, oxidation signals of NA bases are mostly measured using solid electrodes. Besides carbon electrodes, which are undoubtedly most frequently used for label-free DNA detection in biosensors, other solid electrodes, for example, silver,[194,195] platinum,[152] copper,[169] or gold,[171] were employed to a lesser extent. Moreover, liquid mercury was used to study oxidation of G reduction product. G signals at carbon electrodes were, for example, used to monitor telomerase activity[196] or DNA damage induced by radiation,[197,198] by chromium,[199] peroxynitrite,[200] etc. Mechanistic aspects of bases oxidation were recently reviewed.[32,201]

3.2.2.1. Carbon Electrodes. There are plenty of papers concerning NA electrochemistry on carbon electrodes, including many reviews.[27,29,71,73,74,76−80,202−207] Various carbon materials were employed in NA analysis, including highly oriented pyrolytic graphite (HOPG), basal or edge plane pyrolytic graphite, pencil graphite, screen-printed carbon strips, CPE, GCE, BDD, highly conducting diamond films, graphene, carbon nanotubes (CNTs), etc. Some of these materials were compared in a single study, showing that CPE, pyrolytic graphite, HOPG and carbon strips are suitable for analysis of trace amounts of DNA; carbon fiber and glassy carbon required higher DNA concentrations.[208]

Oxidation of A and G residues at carbon electrodes was first observed in the late 1970s,[139,209] yet with inferior sensitivity as compared to the mercury electrodes. Carbon electrodes challenged the mercury electrodes in terms of sensitivity only after the application of constant current chronopotentiometric stripping (CPS) analysis or SWV with baseline correction, enabling determination of submicromolar ODN concentrations.[210,211]

Carbon nanotubes have brought further advancements into the rapidly evolving field of DNA sensors. A contribution of CNTs lies mainly in their unique electric, thermal, chemical, mechanical and 3-D spatial properties.[82,212] Besides papers dealing with DNA hybridization detection using enzyme-labeled ss probe attached to the CNT (section 5.3), several articles were devoted to direct oxidation of DNA G residues on CNT.[213−219] The method relies on the enhancement of the G oxidation due to the large surface of the CNTs, accommodating an increased number of NA molecules. More details regarding the use of CNTs can be found in section 5.3.

Graphene is another promising material for analysis of intrinsic oxidation signals of the DNA.[160,220−223] Although originally prepared by exfoliation (repeated peeling) of HOPG,[224,225] the most economical way to prepare graphene is now considered chemical[226] or thermal[227] reduction of graphene oxide. For instance, Zhou et al. used chemically reduced graphene oxide on a GCE to determine all four DNA bases at physiological pH without a need for prehydrolysis step and detected single nucleotide polymorphism (SNP) in short ODNs.[160] Analogically to previous voltammetric determination of all four nucleotides performed at GCE,[158] concentration of DNA was still rather high (10 μg/mL). It was recently shown that besides reduced graphene oxide, an anodized epitaxial graphene could be applied for DNA analysis. For instance, differentiation between dsDNA and ssDNA, resolution of DNA from uric acid or dopamine, or discrimination of SNPs, were demonstrated.[220,223]

3.2.2.2. Mercury Electrodes. As mentioned above, oxidation signals of DNA can be obtained also at mercury electrodes.[20,97] When the mercury electrode was exposed to highly negative potentials (∼ −1.8 V in aqueous media and at neutral pH), G residues in an adsorbed NA molecule were reduced. Although during a cathodic scan the G reduction merged with the background discharge, G reduction product (7,8-dihydroguanine) remained at the electrode and was reoxidized back to G in an anodic scan, yielding an oxidation peak (peak G; ∼ −0.3 V). Under suitable conditions, the process was shown to be chemically reversible, supported by a constant height of peak G during repeated scans in cyclic voltammetry (Figure 5).[228]

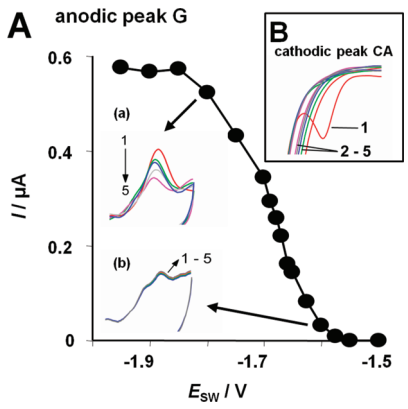

Figure 5. Cyclic voltammetry of DNA at HMDE. (A) Dependence of anodic peak G (around −0.3 V) on switching potential, E_{SW}. (a) 5 repetitive scans (1−5) at E_{SW} −1.8 V showing gradually decreasing peak G; (b) 5 repetitive scans at E_{SW} −1.6 V showing no decrease of the peak G. (B) 5 repetitive scans displaying cathodic peak (A and C reduction) at negative potentials (around −1.5 V), suggesting that reduction of A and C residues was irreversible (peak CA disappeared after the first scan as a result of blocking of the electrode by the DNA reduction product). In contrast, peak G was chemically reversible. Adapted with permission from ref 228. Copyright 1986 Elsevier.

Besides CV, other methods were employed (such as SWV), consisting of a brief application of potential around −1.8 V, followed by scanning to positive potentials.[229] Peak G was symmetrical, sufficiently distant from the background discharge and was used for determinations of low DNA concentrations (tens of nanograms of chromosomal DNA per milliliter).

3.2.3. Changes in DNA Structure. As mentioned in the beginning of this chapter, it was possible to distinguish dsDNA from ssDNA by DC polarography already about 50 years ago.[100,112] At relatively high DNA concentrations (<0.5 mg/mL), chromosomal, thermally denatured ssDNA produced DC polarographic wave close to the background discharge, while native dsDNA was inactive (Figure 3A).[112] Reduction wave of ssDNA was poorly developed and more sensitive methods were needed. With the application of DPP, micromolar and submicromolar concentrations of ssDNA (related to the monomer content) were detectable.[123] ssDNA produced two DPP peaks: peak I (∼ −1.2 V) of capacitive nature, and peak III (between −1.4 and −1.5 V), attributed to the reduction of A and C (forming a single peak; Figure 4B). Reduction was irreversible and proceeded in a protonated state.[104] dsDNA produced capacitive peak I, followed by much smaller peak II (∼ 80 mV more positive than peak III of ssDNA), but no peak III appeared. Peak II was assigned to open regions in double-helical structure, such as at single-strand breaks (ssb) or ends of the molecules, as documented by absence of peak II in covalently closed circular (supercoiled) DNA (scDNA) at room temperature.[230] Viral dsRNA (assuming the A-form) displayed DPP responses similar to those of dsDNA (in the B-form).[231] Denatured form of RNA yielded high peak III similar to that of denatured DNA (Figure 4B).

The primary reduction sites of A and C at mercury electrodes are located in the interior of the DNA double helix, forming a part of the Watson−Crick hydrogen bonding system. The primary reduction site of G at mercury as well as the primary oxidation sites of G and A at carbon electrodes (Figure 4A) are not involved in hydrogen bonding, being situated closer to the surface of the molecule. In agreement with the location of the A and C reduction sites participating in Watson−Crick hydrogen bonding, the DNA reduction currents show a high sensitivity to the DNA structure. At carbon electrodes, the difference between the peak G heights of ss and dsDNA was substantially smaller (Figure 4E) as compared to differences in peak III (measured with DPP/DPV at Hg electrode), in agreement with location of the G oxidation site close to the molecule surface.

The great differences in the DPP responses of ds and ssDNAs were utilized in the 1960s and 1970s to study changes in the DNA conformation including small damage of dsDNA by various chemical and physical agents,[118] DNA melting and premelting, etc. These experiments required concentrations of at least hundreds of micrograms/mL of the dsDNA and tens of micrograms of ssDNA to obtain well-developed peaks.

3.2.4. Adsorptive Stripping Techniques. To increase sensitivity of the EC assays for NAs, adsorptive stripping (AdS) technique was employed, making use of a strong NA adsorption at the surface of the HMDE. By stirring, the NA molecules were faster transported and accumulated over a longer time (than at DME) at the HMDE surface, resulting in an increased reduction or oxidation signals from NAs adsorbed at the electrode. Application of DPV or linear sweep voltammetry (LSV) stripping for measuring reduction signals of A and C residues did not yield the desired increase in sensitivity, because at low concentrations the cathodic peaks merged with the background discharge.[125] Better results were obtained using AdS cyclic voltammetry (CV) for measuring anodic peak G at more positive potentials (∼ −0.3 V),[232] or AC voltammetry (ACV) yielding nonfaradaic (tensammetric) signals.[233] Although the sensitivity was improved by 2 orders of magnitude, rivaling the gel electrophoresis for the first time, the sample volume used in the experiments was still relatively large (≥1 mL). Recently, a well-developed cathodic peak III_{SW}

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was obtained with AdS SWV (Figure 4C), comparable to DPP peak III (Figure 4B). Using peak III_{SW}, a better sensitivity was obtained than with anodic peak G.[193] This peak, although little less developed than at the HMDE, was produced also at solid amalgam electrodes (Figure 4D).[193]

AdS in combination with elimination voltammetry with linear scan (EVLS) was shown to resolve overlapping reduction peaks of C and A by eliminating kinetic and charging currents, while conserving the diffusion current. EVLS was applied for studies involving not only short ODNs,[234−237] but also chromosomal DNAs.[238] A heptamer d(GCGAAGC), forming a stable hairpin-like structure, was also studied by EVLS and was detected down to 2 nM concentration.[239] More work will be necessary to show whether EC analysis of short ODNs is sufficiently sensitive to the DNA structure to recognize a hairpin from a linear ODN. In difference to long dsDNA molecules in which "fraying" of the DNA ends at the electrode surface can be neglected (section 4), in short ODNs opening of base pairs at the end of the molecule may play significant role. Theory and practice of EVLS were reviewed.[240−242]

3.2.5. DNA-Modified Electrodes. Amount of DNA in one mL samples was still rather high, compared to that of the gel electrophoresis commonly used for the studies of plasmid and viral DNAs. Attempts were therefore made to decrease the volume of DNA samples required for the EC analysis. Considerable reduction in the volume was achieved by adsorbing NA molecules from small drops of the NA sample (3−5 μL), followed by a transfer of the NA-modified electrode to a blank electrolyte.[125,142] The technique named adsorptive transfer stripping (AdTS, ex situ, Figure 6) made possible

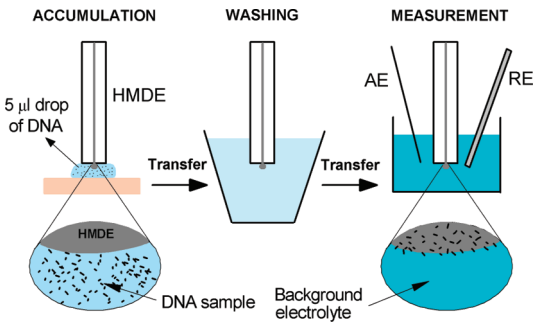

Figure 6. Simplified scheme of adsorptive transfer stripping technique (AdTS, ex situ). DNA is adsorbed from small, microliter-sized DNA-containing drop of solution, and accumulated at HMDE. The DNA-modified electrode is then washed, followed by the electrode transfer into a blank background electrolyte for EC measurement. AdTS technique is applicable also to other electrodes, including SAE or carbon electrodes. Adapted with permission from ref 188. Copyright 1996 Wiley−VCH.

determination and characterization of NA samples usually present in low quantities, for example, plasmid and viral DNAs and their fragments, synthetic ODNs, etc. The adsorbed layer made of NA molecules was relatively stable, resisting washing, providing thus first DNA- or RNA-modified electrodes. The signals obtained by AdTS technique did not substantially differ from the conventional AdS. It was shown that after the transfer, dsDNA retained its conformation and could be distinguished from ssDNA.[125]

AdTS also offered additional advantages, for instance (a) many low MW substances but not DNA could be easily washed away during the transfer, and thus their interference was suppressed; (b) the separation of the adsorption medium from the background electrolyte made it possible to separately optimize conditions for the adsorption and for the EC measurement; (c) it was possible to study interactions of the DNA-modified electrode with other molecules present in solution without being influenced by DNA interactions in bulk solution.[125,142] AdTS was later shown to be effective also at carbon electrodes.[243]

3.2.6. Solid Amalgam Electrodes. Despite some unique features of liquid mercury electrodes, including atomically smooth surface, easy surface renewal (and thus excellent reproducibility of a clean electrode surface) and highly negative potential window, there is a tendency to avoid their use in DNA sensing due to low mechanical stability (complicating its application to flow-through systems or portable sensors), and also due to a fear of toxicity. Number of experiments with metals or simple compounds has shown that solid amalgam electrodes (SAE; or alternatively metal SAE, Me-SAE, where Me is Ag, Au, Cu, Ir, etc.) could be successful substitutes.[244−246] There are more types of SAEs, depending on their surface conditions. These comprise polished (SAEs not containing liquid mercury; p-SAE), meniscus-modified (with mercury meniscus; m-SAE), mercury film-modified (MF-SAE), composite (with solid polymers), or paste amalgam electrodes (amalgam powder in pasting liquid).

Many of these SAEs were applied to study NA components,[176,177,247] ODNs[248] and plasmid[177,249] or chromosomal DNAs.[175,176,193,249,250] DNA adsorption at the SAE surface is strong enough to resist washing, thus allowing AdTS analysis of small sample volumes.[251] The m-AgSAE was used as a detection tool of enzymatic or chemical DNA cleavage, serving as a sensor for DNA nicking substances,[251] or as a detection electrode in double-surface technique hybridization experiments (see Section 5.1 for more details), allowing determination of the length of repetitive DNA sequences.[250] Furthermore, the MF-AgSAE and p-AgSAE allowed differentiation between sc and linear DNA and were successfully applied for the detection of DNA strand breaks induced by ionizing radiation.[249] Recently, SAEs were used also in analysis of thiolated monolayers[252] or proteins[253,254] and first chips with SAEs were introduced.[255]

3.3. Adsorption of Nucleic Acids and Their Components at Electrodes

EC behavior of NAs at an electrode/electrolyte interface relies on the strong physical adsorption of NAs at mercury (both liquid and solid amalgam) and carbon electrodes. Adsorption of NAs to bare gold is weaker, but not negligible:[256] gold surface is almost exclusively used for covalent attachment of thiol-modified ODNs (due to a strong bond between Au and S), forming self-assembled monolayers (SAMs) widely applied in DNA hybridization sensors.[50,257,258] High-density pinhole-free SAMs of thiolated ODNs were recently observed at HMDE.[259]

In this Section, only physical adsorption of unmodified NAs will be discussed. Immobilization of thiol-modified ODNs on Au electrodes was reviewed,[257] and it is mentioned in sections 5.5, 5.6, and 6.3. Literature on different immobilization techniques requiring modification of NAs or electrodes, using for example, (strept)avidin−biotin system, nanoparticles or conducting polymers, can be found elsewhere.[57,63,260−267] For characterizing adsorption of various biomolecules at electrode surfaces, electrochemical impedance spectroscopy (EIS), which measures the impedance of the electrode double layer as a

function of the frequency, is a method of choice. Adsorption of the biomolecules, including DNA, leads to a change of capacitance and interfacial electron transfer resistance. More details regarding the use of EIS in current DNA biosensors can be found in section 5.2.2 or in review articles.[37−40,268]

3.3.1. Adsorption at Mercury Electrodes. Early insights into the interfacial behavior of NAs and its components were provided by differential capacitance measurements with NA solutions at DME.[115,116] Since the aqueous solvents usually possess much higher dielectric permittivity than NAs, differential capacitance decreases when the water molecules and ions are displaced by NAs (or their components) at the surface of the electrode. NAs, bases, nucleosides, and nucleotides: they all were shown to strongly adhere to the mercury surface.[27,28,119,120,269−274]

Generally, adsorption of NAs (and other biopolymers) is more complicated than that of low MW compounds. In a process called two-dimensional condensation, NA bases form a compact self-assembled film with monomolecular thickness, in contrast to other pyrimidine and purine derivatives not occurring in NAs, which lack such ability.[119,270] 2-D condensation is manifested by a capacitance "pit" on capacitance−potential (C−E) curves, being sensitive to the presence of solvent ions (Figure 7). Forces maintaining a stability of the

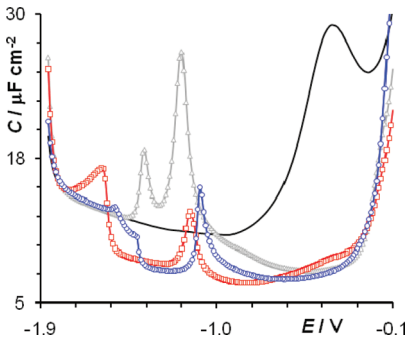

Figure 7. C−E curves of dT_{30} (blue), dC_{30} (red), and dA_{30} (gray) measured at the HMDE with ex situ technique. The C−E curves were measured by potential scan from −0.1 V to more negative values. Prior to the potential scans, the ODNs were adsorbed at the HMDE for 8 min at open current potential from 10 μL drop of 1 μM solution of the ODNs. Black line represents a background electrolyte. Adapted with permission from ref 121. Copyright 2008 Elsevier.

DNA double helix (i.e., hydrogen bonds and stacking interactions) are similar to those that drive 2-D condensation of NA bases on the mercury surface.[120,122,275,276] If the base is neutral, capacitance pit is formed close to the potential of zero charge (pzc); in the case of the base carrying positive charge (e.g., C at acidic pH), "pit" potential is shifted toward negative values.[277,278] Up to recently, it was believed that it was only the NA monomeric components that were able to undergo 2-D condensation and form the capacitance pit. Recently, it has been shown that the pits are produced also by pyrimidine, but not by purine 30-mers (Figure 7).[121] More work will be necessary to reveal relation between the 2-D condensation on one hand and lengths and structures of ODNs on the other hand.

Figure 8 shows that adsorption−desorption behavior of dsDNA differ from that of ssDNA.[279] At lower ionic strength, positively charged electrode attracts inadequately shielded negative charges on the phosphate backbone. At negative potentials, unscreened charges are repulsed from the electrode,

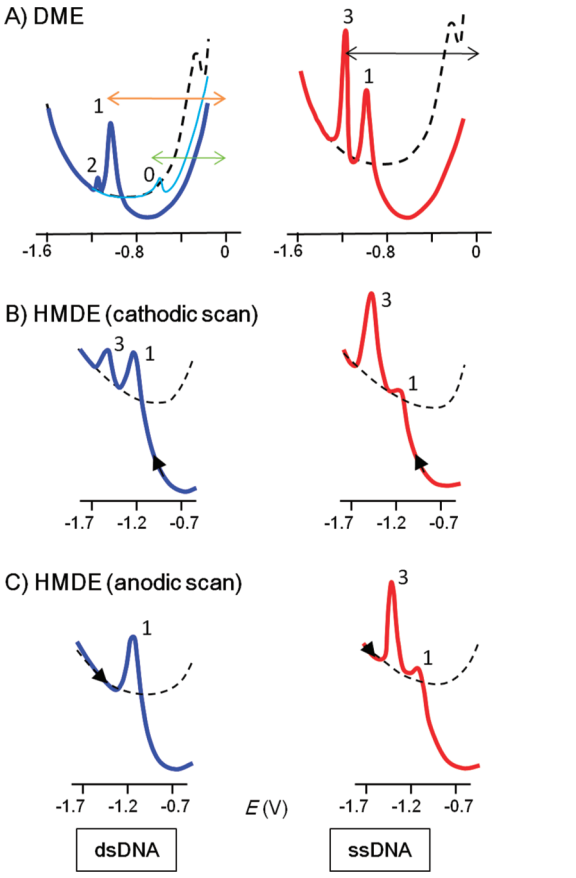

Figure 8. Schematic representation of AC polarograms and voltammograms of ds (left, blue) and ss (right, red) calf thymus DNA. (A) AC polarograms and the role of the DNA constituents in the DNA adsorption at DME. DNA polarograms obtained at moderate (solid bold) and low (solid light) ionic strength; pH 8, background electrolyte (dashed). At low ionic strengths dsDNA is adsorbed mainly electrostatically (via unscreened negatively charged phosphates) at the positively charged electrode (green arrow). At moderate ionic strengths dsDNA can be adsorbed via its backbone and some accessible base residues even at a negatively charged electrode (orange arrow). ssDNA is adsorbed via bases in a broad potential spectrum (black arrow). (B−C) AC voltammograms at HMDE presented as adsorptive stripping admittance curves of ss and dsDNA with negative- (B) and positive-going (C) scans, as indicated by arrows at the curves. Peak 1 is adsorption/desorption peak related to sugar−phosphate backbone and DNA reorientation, peak 2 is related to adsorption/desorption of some open regions in dsDNA and peak 3 is corresponds to adsorption/desorption of DNA segments adsorbed via bases in ssDNA. (A) Adapted with permission from ref 283. Copyright 1995 Academic Press. (B) Adapted with permission from ref 284. Copyright 2000 Wiley−VCH.

characterized by adsorption/desorption (tensammetric) peak. At higher ionic strengths, nonelectrostatic (hydrophobic) forces prevail. Under these conditions, dsDNA displayed a desorption peak at less negative potentials (peak 2 at ∼−1.2 V vs SCE) than ssDNA (peak 3 at ∼−1.4 V vs SCE). The former peak resulted from adsorption/desorption of dsDNA segments containing small number of accessible base residues, while peak 3 was due to desorption of ssDNA adsorbed to the electrode stronger via hydrophobic bases.

Peptide nucleic acid (PNA) with neutral backbone[280] withstood exposure to highly negative potentials (under conditions sufficient to remove negatively charged DNA with the same

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sequence), as shown by AC impedance measurements.[281,282] Such a strong adsorption was attributed to strong hydrophobic interactions of PNA with the surface and also to lateral interactions between the PNA molecules.

3.3.2. Adsorption at Carbon Electrodes. Nucleic acids are firmly and irreversibly adsorbed also at carbon electrodes, as first demonstrated more than three decades ago.[139] Because of a hydrophilic nature of the carbon surface (reached e.g., by an oxidative pretreatment), NAs are adsorbed via phosphate backbone, leaving bases accessible for hybridization. If the carbon electrode is positively charged, electrostatic attraction of negatively charged backbone makes the adsorption even stronger. Analogically, a shift toward more negative potentials results in DNA desorption caused by an electrostatic repulsion. Besides accumulation potential, other parameters, including ionic strength or a type of the background electrolyte, influence the adsorption behavior of DNA at carbon electrodes.[285] In contrast, neutral PNA showed different behavior. The adsorption of PNA at carbon electrodes is characterized by increased surface packing, especially due to intermolecular surface interactions, and by a weaker adsorption at positive potentials, as compared to DNA.[285]

4. POTENTIAL-DRIVEN CHANGES IN CONFORMATION OF SURFACE-ATTACHED DNA

4.1. Introduction

Large differences between the DPP responses of ss- and dsDNAs (Figure 4B) suggest that the double-helical structure of the dsDNA is not significantly disturbed at the DME surface. On the other hand, SW voltammograms at HMDE (Figure 4C) show smaller differences between ss- and dsDNAs; in addition to peak II_{SW}, dsDNA displays a small peak III_{SW}, indicating partial unwinding of the dsDNA at the electrode surface. Changes in DNA structure at electrically charged surfaces are of great interest both from the surface chemistry and biological points of view. As early as in 1961, Miller assumed that at positive potentials a partial unwinding of dsDNA took place on the electrode, while at negative potentials the DNA double-helical structure was preserved.[115,116] Later, Flemming[286] did not confirm Miller's results and concluded that DNA retained its double-helical structure regardless of the HMDE potential. In 1974 it was shown independently by Palecek (who worked with NPP at DME and neutral pH)[131] and by Nurnberg (using LSV with HMDE at weakly acidic pH)[132] that DNA is unwound at mercury electrodes. At neutral pH, heights of NPP waves of native dsDNA depended on the initial potential, E_i, showing the highest values around E_i −1.2 V (unwinding potential region U), while the heights of denatured ssDNA were almost independent of E_i (Figure 9).[131] This finding was explained by partial denaturation/unwinding of the dsDNA due to prolonged exposition to potentials of region U. In contrast to NPP (working with large voltage excursions, holding the electrode at the initial potential for a substantial part of the drop lifetime),[287] DPP (working with small voltage excursions) did not show any dependence of dsDNA peaks on E_i and produced results in a good agreement with studies of DNA in solution by optical and other methods. These results suggested that it was the prolonged exposition of dsDNA to certain E_i values, which was responsible for the observed changes in NPP signals of dsDNA.

For years, Berg and Flemming did not believe that DNA could be denatured at the electrode surface.[118,135,290] Originally, they assumed that at potentials positive to pzc the DNA surface

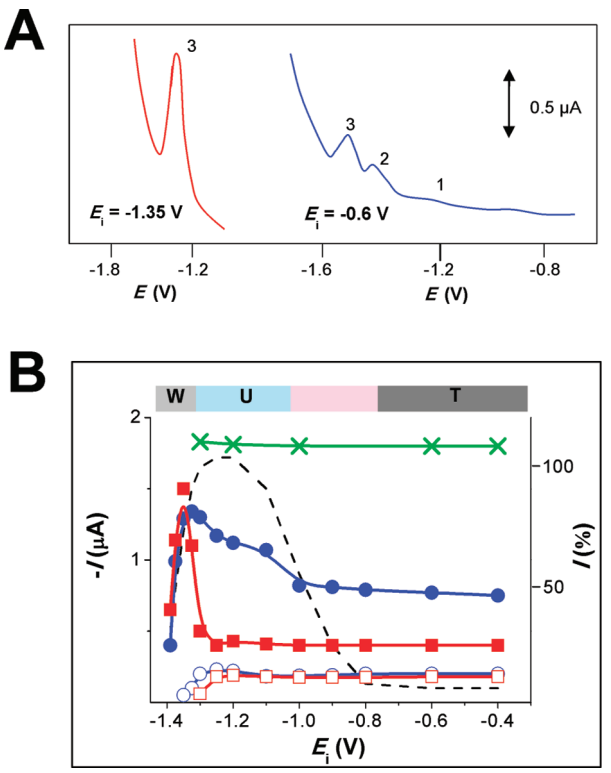

Figure 9. Effect of nucleotide sequences in biosynthetic polydeoxynucleotides on the double helix opening at the electrode surface. (A) Voltammetric stripping peaks of poly(dA-dU)·poly(dA-dU) at two initial potentials, E_i: −0.6 V (blue) and −1.35 V (red). At E_i corresponding to region T, small peaks 2 and 3 are observed; at E_i of region U, peak 2 (characteristic for dsDNA) disappears and peak 3 (typical for ssDNA) strongly increases. (B) Dependence of height of (i) peak 2 of poly(dA-dU)·poly(dA-dU) (red, □) and poly(rA)·poly(rU) (blue, ○), and (ii) peak 3 of poly(dA-dU)·poly(dA-dU) (red, ■), poly(rA)·poly(rU) (blue, ●), poly (rA) (green, ×) and calf thymus dsDNA (black dashed). The DNAs were adsorbed at HMDE at the given E_i for 60 s, followed by potential scanning at 0.5 $V.s^{-1}$. Peak height of calf thymus dsDNA is expressed in per cents of the height of thermally denatured calf thymus DNA (right axis). Concentrations: synthetic polynucleotides (50 μM, related to phosphorus content); calf thymus DNA (100 $\mu g.mL^{-1}$). Background electrolyte 0.3 M ammonium formate, 0.05 M sodium phosphate, pH 6.9. **T** is the potential region where fast opening of short regions of the DNA double helix takes place. In region **U** relatively, slow DNA opening occurs, involving large portion of the DNA molecule. In region **W**, no opening of the duplex DNA was detected. DNA data and more details about potential regions can be found in previous papers.[288,289,295] Adapted with permission from ref 288. Copyright 1985 Slovak Academy of Sciences.

concentration was low (loosely packed layer at full electrode coverage), while at more negative potentials this concentration and the thickness of the adsorbed layer increased as a result of changes in orientation of the adsorbed DNA molecules from flat to perpendicular. Further, they speculated that segments of the DNA molecules extending into the solution interacted with each other, forming a compact layer.[135] Perpendicular orientation of very long chromosomal DNA molecules was however rather improbable. Moreover, great increase of the DNA surface concentration required adsorption of additional DNA molecules at the negatively charged electrode from the bulk of solution. Ex situ experiments (with the dsDNA-modified electrode immersed in a blank background electrolyte) yielded similar results[291] as conventional experiments with the electrode immersed in the DNA solution (Figure 9).[118,289]

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Under the conditions of the ex situ experiments, adsorption of additional DNA molecules from the bulk was not possible (because of absence of DNA in solution) and the large increase of DNA concentration at negatively charged electrode was difficult to imagine. Later, Berg speculated that DNA is at Hg electrode in the so-called π-state involving A-form and condensation of DNA.[290,292] This concept lacked experimental support and the assumption that the observed changes could be due to the DNA B to A transition were at variance with EC responses of dsRNA in A-form, which did not significantly differ from that of the B-form dsDNA.[27,118,231,288]

At present, ample evidence is available showing that dsDNA and dsRNA can be unwound at the negatively charged surfaces[118,131,132,291] and that such surface denaturation can be observed both with different types of DNA and RNA molecules including very long chromosomal DNAs,[27] viral[231] and biosynthetic NAs[288] as well as with much shorter synthetic ODNs (section 4.3). First experiments aimed on evaluating the effect of electrode potential on the surface-immobilized DNA were done with DNAs noncovalently bound to the electrode surface.[27,28] This type of NA research has continued up to the present time.[293] Only in the recent decade the researchers' attention turned to synthetic ODNs covalently bound to the electrodes. We believe that both types of the NA surface binding studies can bring important information about the structure and properties of surface-immobilized NAs. Moreover, research into oligonucleotides covalently bound to the electrode surfaces can be directly utilized in the development of the DNA sensors. To our knowledge, unwinding of NA molecules at electrode surfaces has not been yet summarized in any journal review. On the other hand, some reviews were included within book chapters on NA electrochemistry.[27,28] Here, we wish to summarize the decades of research into the unwinding of DNA noncovalently bound to negatively charged electrode surfaces and to review the recent progress in studies of ds oligonucleotides covalently bound to electrodes.

4.2. NA Molecules Noncovalently Bound to Negatively Charged Surfaces

4.2.1. Long DNA and RNA Molecules. At neutral pH, long DNAs produce at Hg electrodes three LSV peaks (Figure 9A). Peak 1 is of capacitive nature responding little to changes in the DNA structure. Peak 2 is characteristic for dsDNA and disappears as a result of the DNA denaturation (e.g., thermal denaturation in solution). Peak 3 is characteristic for ssDNA and it is absent in intact linear DNA, which was not exposed to potentials of the region U (e.g., in DPP in combination with DME). On the other hand, this peak is usually produced by dsDNA which was even briefly exposed to potentials of the region U. Peak 3 requires free accessibility of bases in ssDNA, while peak 2 is related to the reduction of a limited number of accessible bases at the DNA ends, at the strand breaks and other DNA damage sites.[27,28,118,294] LSV of linear dsDNA produced at HMDE both peaks 2 and 3. When instead of HMDE the DME in combination with DPP was used, no peak III was produced by the same dsDNA (Figure 4B) because at DME, the exposure of DNA to the potentials of region U could not induce peak III appearing at potentials more negative than those of region U.

DNA unwinding starts from ends of the DNA molecule, including those resulting from the ssb formation.[27,28,294] DNA opening at Hg electrodes is relatively slow process[291] (about 90% of a chromosomal DNA is opened in ~100 s) and its rate increases with shifting of the electrode potential to more negative values.[295] The DNA duplex opening is partially irreversible[131,296] and depends on the DNA nucleotide sequence.[288] In calf thymus DNA, both GC and AT pairs are involved in the early stage of the opening process.[131,296]

4.2.2. Biosynthetic Double-Stranded Polynucleotides. Biosynthetic ds polynucleotides with different nucleotide sequences, including homopolymer pairs, such as poly(dA)·poly(dT), poly(rA)·poly(rU), and poly(dG)·poly(dC) and alternating sequences of poly(dA-dT)·poly(dA-dT), poly(dA-dU)·poly(dA-dU), poly(dG-dC)·poly(dG-dC)[288,297] were studied by voltammetry with HMDE. With the exception of poly(dG)·poly(dC)[298] these duplexes showed distinguished regions U (Figure 9).[288,295,299,300] Duplexes with alternating sequences displayed a very narrow region U (half-width <100 mV) and the rate of opening of the double helix strongly depended on the electrode potential within the region U (Figure 9B). In the homopolymer pairs, the width of region U was comparable to that of natural DNAs (>200 mV) and it was composed of two distinct phases. The differences in the interfacial behavior of duplexes with alternating sequences on one hand and homopolymer pairs on the other hand were explained by nonequal adsorbabilities of purine and pyrimidine chains in the homopolymer pair molecule, in contrast to equal adsorbability of both chains in alternating sequence polynucleotides.[288]

4.2.3. Carbon Electrodes. DNA unwinding was reported not only on mercury but also at graphite electrodes.[189,301] Voltammetric oxidation signals of G and A residues in dsDNA were increased due to exposing the electrode to sufficiently negative E_A values (between −0.4 and −0.8 V).[189,301]

4.2.4. Closed Circular Duplex DNA Molecules. In addition to EC studies of linear DNA and RNA molecules, also EC responses of closed circular duplex DNA (cdDNA) were investigated.[230,302−307] cdDNAs are present in various organisms but among them plasmid scDNA molecules have been most frequently used in biochemical and molecular biological research (see also section 6.2.2).[308] Plasmid DNAs of different MW can be easily isolated. They usually contain supercoiled tertiary structure at native superhelix density.[308,309] scDNAs containing about 3000 base pairs have been frequently used. sc and cdDNAs do not contain any molecular ends and strand interruptions. Extensive unwinding of scDNA in solution (under the conditions inducing denaturation of linear DNA) is prevented for topological reasons.[310,311] Introduction of a single interruption into the sugar−phosphate backbone of scDNA results in formation of an open circular (oc) DNA molecule, which is relaxed (free of the DNA supercoiling) and can be denatured (unwound) in solution, similarly to linear DNA.

It was thus interesting to study EC responses of plasmid DNAs after their exposition to potentials of region U and T (Figure 9B). Exposure of the scDNA to the potentials of the region U at the HMDE surface resulted in no detectable DNA opening, as indicated by absence of capacitive[303] and faradaic[302] peaks 3 (produced by ssDNA) in agreement with the limitations in unwinding of scDNA in solution.[303,310] Similarly, covalently closed circles of cdDNA, free of supercoiling (in which unwinding in solution is also prevented), produced no AC impedance peak 3 (Figure 10A).[281,304] Introduction of a ssb in scDNA (e.g., by γ-irradiation or enzymatically, using DNase I) resulted, however, in AC impedance peak 3 (Figure 10A), suggesting that a substantial portion of bases in the ocDNA molecule interacted with the HMDE surface. On the other hand, the same ocDNA produced no peak 3 on the DME (Figure 10B) suggesting absence of an appreciable amount of

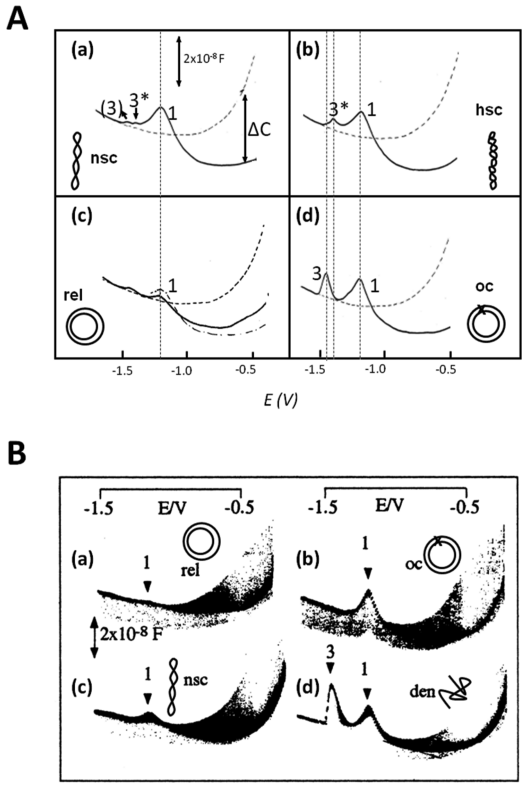

Figure 10. Sections of *C*−*E* curves at hanging mercury drop electrode (HMDE) and dropping mercury electrode (DME) modified with plasmid pUC19 DNA. (A) HMDE: (a) negatively supercoiled DNA at native superhelix density (nsc); (b) highly supercoiled DNA (hsc $-\sigma \geq 0.11$); (c) scDNA relaxed by topoisomerase I (rel); (d) open circular DNA (oc). DNA concentration was 100 μg/mL, background electrolyte (dashed). Curve (− • −) in (c) was DNA at 250 μg/mL. ocDNA was prepared from covalently closed circular DNA by γ-irradiation. In (a), the meaning of the capacitance decrease (ΔC) is displayed. (B) DME. (a) topoisomerase I-relaxed DNA; (b) open circular DNA; (c) native supercoiled DNA; (d) linear denatured DNA (den). DNA concentration was 200 μg/mL. Note: Compared to HMDE, at DME the DNA is adsorbed again and again at each mercury drop. The DNA, which is adsorbed at DME charged to potentials of peak 3, cannot be denatured at the mercury drops whose potentials are within the region W and out of region U (see Figure 9B). In region W, dsDNA is weakly adsorbed and tends to desorb.[295] On the other hand, DNA which is adsorbed at Hg drops (on DME) exposed to the potentials of region U cannot yield peak 3, because these drops fall down at potentials more positive than those of peak 3. Adapted with permission from ref 304. Copyright 2008 American Chemical Society.

bases capable to interact with the electrode. In contrast to differences between responses of ocDNA at DME and HMDE, voltammetric responses of scDNA obtained with DME and HMDE did not qualitatively differ. Similarly, denatured DNA yielded qualitatively the same responses at these two electrodes. Clearly, DME and HMDE yielded similar results with DNAs, in which (a) bases were accessible (e.g., in ssDNA) or (b) were hidden in the interior of the DNA duplexes and DNA unwinding was prohibited (e.g., in cdDNA). Linear DNA cross-linked with bifunctional platinum complexes (preventing DNA strand separation) also resisted the double helix unwinding within the region U, but not by other types of DNA modification by some Pt anticancer drugs.[312] It has been shown that covalent bonds (cross-links) between the DNA strands limit or prevent DNA unwinding in solution.[308] These results suggest that DNA containing free ends (but no cross-links) can be partially denatured due to a short exposure to negatively charged electrode surface. In contrast, covalently closed circular DNAs (in which DNA unwinding in solution is prevented for topological reasons) do not denature at the electrode surface.

We may conclude that absence of peak 3 on the *C*−*E* curves of ocDNA obtained with DME (Figure 10B) was in agreement with solution structure of ocDNA, in which almost all bases should be included in an intact B-DNA structure and not accessible for interactions with the environment. Presence of peak 3 on curves of the same ocDNA obtained with HMDE suggested that appreciable amount of base residues interacted with the HMDE surface. This can be explained by exposition of bases in ocDNA (having no topological restrains in the DNA unwinding) to the solvent during the potential scanning through the region U.

4.2.5. DNA Surface Unwinding and Detection of DNA Damage. The above-mentioned ability of EC methods to detect one ssb among a large number of intact sugar−phosphate bonds in scDNA was utilized in the development of sensors for DNA damage.[303,305,306,313−317] Using ACV in combination with HMDE, it was possible to detect one ssb among more than 2×10^5 nucleotides (i.e, one ssb in about 1% of the scDNA molecules).[303] It was shown that the lesions can be detected in DNA down to femtomole level.[318] In this respect, the EC determination was well competitive with a number of currently used DNA damage assays, such as ^{32}P postlabeling,[319] ELISA[320] or mass spectrometry.[321] scDNA can easily be immobilized at the HMDE and the scDNA-modified electrode can serve as a simple biosensor for detection of ssb DNA or for detection of DNA-cleaving agents (Figure 11).[303,305,306,313−316] This type of biosensors was applied both for laboratory-prepared model samples as well as for various "real" specimens.[305] The scDNA-modified electrode was also utilized as a tool for *in situ* monitoring of DNA cleavage by electrochemically generated reactive oxygen species (ROS)[315,322] or intermediates of reduction of chromium compounds.[323] Analogous approach was recently used to monitor ligation - a process inverse to an endonuclease cleavage, utilizing DNA ligase for sealing ssb in DNAs.[324] Using AC voltammetric peak 3, it was possible to differentiate between ligatable and unligatable breaks caused by *E. coli* ligase LigA. Compared to native agarose electrophoresis, which was not able to distinguish more than one ssb per single plasmid DNA molecule (unless DNA structure was disrupted), intensity of ACV peak 3 increased when multiple breaks were present in the DNA molecule. Particularly interesting appear the new detection of damage to DNA bases possessing the high sensitivity of the EC DNA ssb determination. In this method the damage to bases was transformed to strand breaks by DNA repair endonucleases cleaving specifically the DNA at the base damage sites.[187,318] Using this approach, it was possible to detect radiation-induced DNA damage not only in vitro, but also in living cells.[318] More details about EC sensing of the DNA damage can be found in section 7 and other reviews.[29,46,313,317,325]

4.2.6. Effect of Base Ionization. Close to neutral pH and at weakly alkaline pH's, a distinctive region T appears (Figure 9B, 12B). Presence of peak 3 in the region T is indicative of a limited surface denaturation,[289] which is very fast, affecting only a small part of the adsorbed DNA molecule and stops.[294] It is limited to labilized DNA regions such as ends and strand breaks in dsDNA molecules. At pH 5.6, Nurnberg et al. observed only

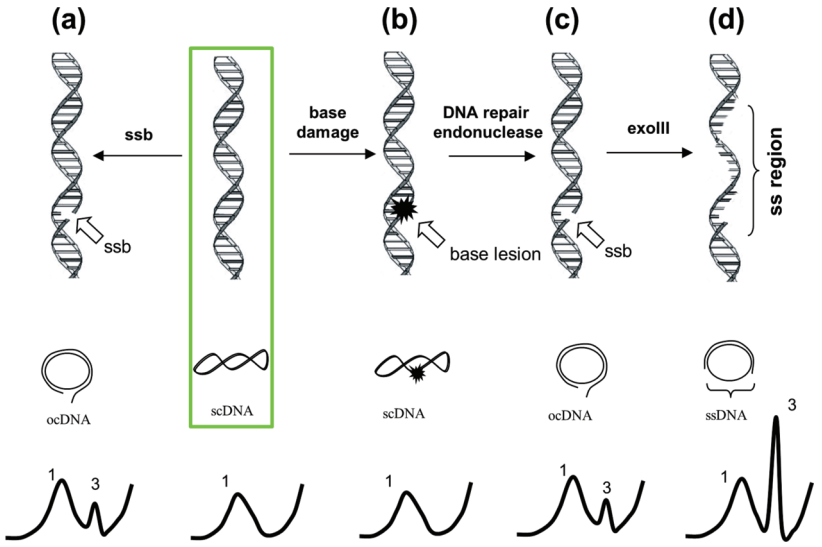

Figure 11. Principles of EC detection of damage to DNA at mercury electrodes. Single-strand breaks (ssb), formed, e.g., by γ-radiation, actions of reactive oxygen species or nucleases in vitro or in vivo (a) transform scDNA (in green rectangle) to ocDNA, yielding peak 3; (b) Formation of small number of base lesions in scDNA molecule (without interruption of the sugar−phosphate backbone) does not significantly change the DNA voltammetric behavior; (c) Some base lesions are recognized by specific endonucleases introducing ssb at the damaged sites, producing peak 3. This peak can be amplified (d) using *E. coli* exonuclease III (exoIII) generating ss regions in oc or linear DNA (but not in intact scDNA). Adapted with permission from ref 317. Copyright 2005 Elsevier.

small dependence of the LSV peak of dsDNA on accumulation potential, without any well distinguished regions U and T.[132,296,326−329] It was shown[299,300] that formation of the distinct T and U regions depended on pH and on the intactness of the dsDNA samples. With a relatively intact calf thymus DNA, marked regions T and U were observed at pH 6.0 (Figure 12A).[299] Decreasing the pH resulted in an increase of the EC signal in the region T, without any significant signal change in region U. These changes were not in accordance with protonation of DNA base residues in solution,[330] suggesting involvement of the electric field-induced shift in DNA protonation at the electrode surface.[331,332]

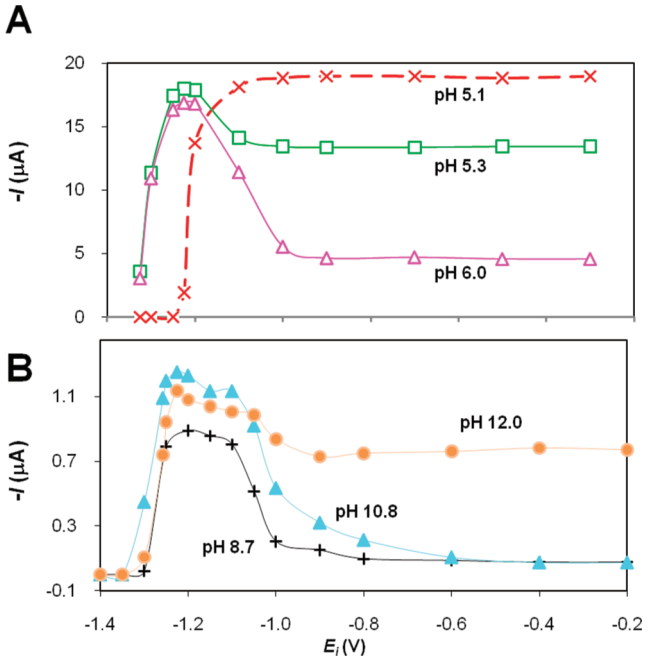

Figure 12. Dependence of heights of dsDNA linear sweep voltammetric peak 3 on initial potential (E_i) at different pH's. Working electrode: HMDE. (A) peak 3 of 420 μg/mL dsDNA at acid pH's: 5.1 (× ; red); 5.3 (□, green) and 6.0 (Δ, magenta) and (B) at alkaline pH's: 8.7 (+, black); pH 10.8 (▲, cyan) and pH 12.0 (●, orange). Potentials were measured against SCE. (B) Adapted with permission from ref 118. Copyright 1983 John Wiley and Sons.

A and C residues in ssDNA are reduced in protonated state.[104,118] At alkaline pH's, DNA is thus nonreducible at mercury electrodes. On the other hand, under these conditions DNA yields capacitive (nonfaradaic) voltammetric signals reflecting changes in the DNA structure similarly to the faradaic signals (observed at neutral pH).[118,333,334] At pH 8.7, the heights of peaks 2 and 3 of native dsDNA changed in dependence on the initial potential (E_i) in a similar way as at neutral pH (Figure 12B). Up to pH 10.8, the well-resolved regions U and T were observed. At pH 12 (where the beginning of DNA alkaline denaturation in the bulk of solution can be expected), the peaks in the region T increased about 10-fold (compared to pH 10.8) and at pH 12, the regions U and T almost merged.

Brabec et al. studied the behavior of native and denatured DNA at DME and HMDE at pH 9.85 in a greater detail.[301] Although they measured at higher ionic strength (0.5) than that shown in Figure 12B, their voltammetric measurements were in qualitative agreement with the results in this Figure. ACV and LSV were used to study native sonicated and unsonicated dsDNA at different frequencies and scan rates, respectively. New data were obtained, supporting earlier views of adsorption of ds- and ssDNAs at Hg electrodes at neutral pH and different ionic strengths (Figures 9 and 12), as well as of surface unwinding of dsDNAs at negatively charged electrode surface.[118] In addition, it was shown that peak 2a, observed when positive voltage scanning was used, was produced only by dsDNA. This peak was earlier reported by Flemming and Pospisil.[335] Brabec et al. suggested that peak 2a can be related to renaturation of DNA, partially denatured at the Hg electrode surface charged to more negative potentials.

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It can be concluded that at alkaline pH's the DNA behavior is in agreement with DNA ionization in solution, showing large changes in the DNA surface unwinding in the region T at pH 12 (Figure 12B), that is, close to the pH of alkaline denaturation of DNA in solution. In contrast, at acid pH's similar changes are observed already at pH 5.3 (Figure 12A), that is, at pH by about 3 units higher than the pH at which acid denaturation of DNA takes place in solution. These results suggest that the electric field-induced shifts in DNA protonation at the electrode may take place at acid pH's, but no significant electric field effect on DNA ionization is observed in region T at alkaline pH values.

4.2.7. Tentative Scheme of the DNA Surface Denaturation. To explain the DNA surface denaturation at mercury electrodes, a tentative scheme was put forward.[27,131,289] At neutral pH and low or moderate ionic strengths, segments of long dsDNA molecules are adsorbed in the vicinity of the pzc via sugar−phosphate backbone as well as via sporadic bases located in the labilized regions of the DNA double helix, for example, at the ends of the DNA molecule or at the strand breaks.

When the electrode potential is shifted to more negative values, such DNA segments tend to desorb. On the other hand, the segments attached to the surface more strongly via a larger amount of hydrophobic bases remain adsorbed at the electrode even at potentials of the region U. Desorption of the segments takes place at more negative potentials, as indicated by capacitive peak 3. At potentials of region U the DNA molecule can be anchored to the surface by an ss segment S_{ads} (e.g., involving one end of the DNA strand), while the adjacent ds segment (not involving significant adsorption via bases) is strongly electrostatically repulsed from the negatively charged electrode. The surface-attached DNA molecule is thus under stress which might result in unwinding of the dsDNA segment (Figure 13). Unwound segment S_{ads} is strongly adsorbed via bases and the DNA surface denaturation proceeds to further regions of the DNA molecule. Considering the length of the chromosomal DNA molecules it can be expected that the unwinding process may take place not only in the inner but mainly in the outer part of the double layer and in the bulk of solution.

4.2.8. Other Surfaces and Detection Methods. Low levels of DNA denaturation at room temperature in the presence of certain types of polypropylene tube surfaces was reported.[336,337] In DNA fragments containing $(GA)_n \cdot (CT)_n$ or $(GT)_n \cdot (CA)_n$ sequences, multimeric complexes were formed. Addition of micromolar concentrations of an ODN prior to adding dsDNA into a polypropylene tube inhibited this surface activity. It was not clear what might attract DNA to the polypropylene surface. It could be expected that DNA in water solution should be repelled from an object with a low dielectric constant such as the polypropylene surface. It was suggested that such a repulsion could be overcome by hydrophobic interactions between the polypropylene surface and the bases of denatured ssDNA,[337] resembling thus strong adsorption of ssDNA on negatively charged hydrophobic mercury surface (section 3.3). In contrast to polypropylene surfaces, no denaturation reaction was observed in borosilicate glass tubes.[337] dsDNA adsorbed on phospholipid membranes adopted an altered conformation resembling DNA denaturation.[338] Opening of the dsDNA at the surface was revealed also by atomic force microscopy (AFM). dsDNA molecule was attached to an AFM tip and the mechanical stability of the DNA double helix was tested.[339] Stretching experiments showed a highly cooperative transition, where the natural B-DNA was converted into a new overstretched conformation called S-DNA. After the B−S transition, a second conformational transition followed due to the DNA duplex melting. Both the B−S and the melting transitions occurred at significantly higher forces in poly(dG-dC) as compared to poly(dA-dT).[340] The above data indicate changes in DNA conformation attached to surfaces, but the nature of these changes appears unclear.

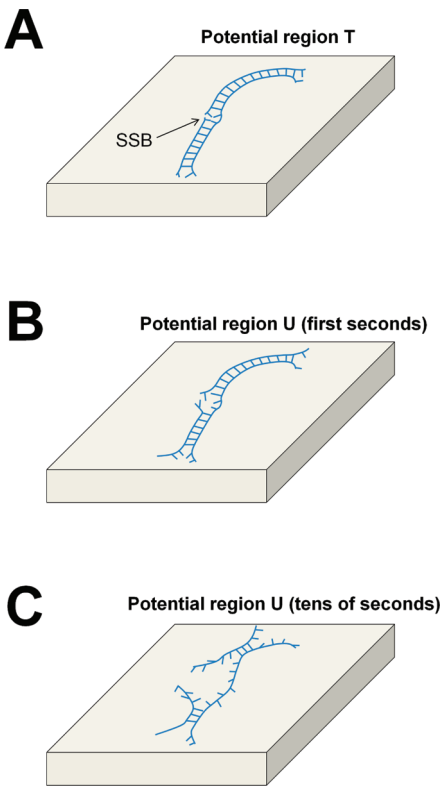

Figure 13. Simplified scheme depicting structural changes in long dsDNA at mercury electrodes induced by an application of negative potential. (A) Limited opening of DNA duplex around the potential of zero charge (potential region T, see Figure 9) near the molecule ends, single-strand breaks (ssb) and other structural disturbances. (B) Slow opening of the DNA duplex in the first seconds after the potential region U (around −1.2 V) is applied. (C) Substantial opening of the DNA duplex after tens of seconds, if the potential is kept sufficiently negative, i.e., in the region U.

4.2.9. Electric Field Effects as Detected by Fluorescence. Microarrays for NA analysis with fluorescence detection are increasingly used in biology and medicine.[50] Briefly, the technique involves attaching microscopic spots representing the ssDNA molecule under study to a surface such as a microscope slide. The slide is then hybridized with a fluorescently tagged solution-phase target and the fluorescent intensity of each spot on the slide is analyzed. The method is, however, not without limitations. One of the main limitations is that the target molecules must be present in high enough concentration to diffuse and react with surface-bound probes. Depending on target concentrations, hybridization times of 12−24 h may be required to obtain an adequate fluorescent signal.[341] Attempts to apply electric field effects to increase the rate of the DNA hybridization have been thus made. It was shown by Sosnowski et al.[293] that hybridization and denaturation of ODNs immobilized on Pt electrodes can be regulated by electric

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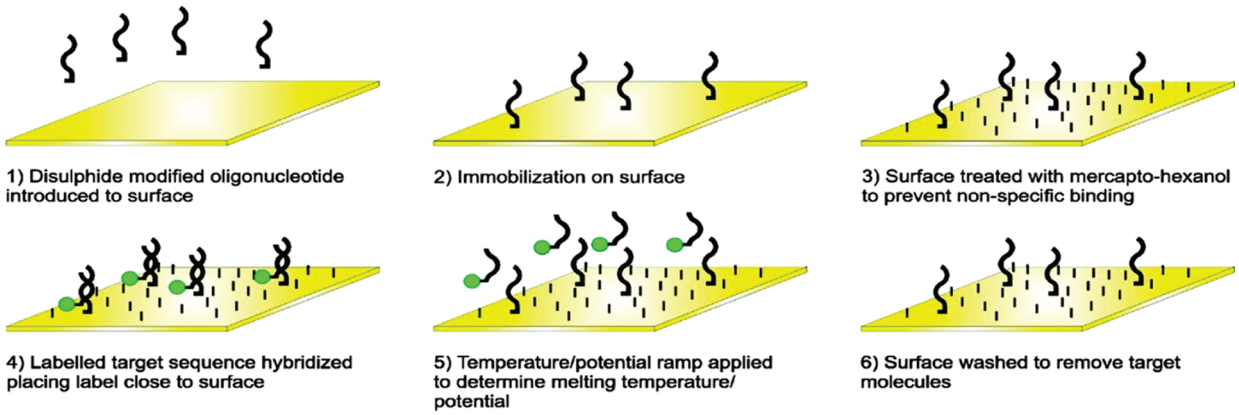

Figure 14. Surface enhanced (resonance) Raman spectroscopy (SER(R)S) at gold surface for monitoring electrically/thermally driven DNA hybridization. The process of detection and characterization of DNA sequences begins with (1) and (2) preparation of the sensing surface followed by (3) passivation with mercaptohexanol to prevent nonspecific binding, (4) detection using SER(R)S-labeled targets, (5) dehybridization with either temperature or potential, and ending with (6) regeneration of the surface for reuse starting again at step 4. The dehybridization process is the key step and yields the information that characterizes mutations. Reprinted with permission from ref 354. Copyright 2008 American Chemical Society.

fields. At positively charged electrodes significant acceleration of the DNA hybridization was observed as compared to neutral (open current potential) test sites. Application of negative potentials resulted in DNA denaturation. With the controlled electric fields, efficient and rapid discrimination of single base pair mismatches was possible. It was concluded that observed stimulation of the hybridization or induction of DNA denaturation as well as the single base mismatch discrimination could hardly be explained by a pure electric field effect because DNA in the permeation layer was too far from the electrode surface. Reduction of the stability of the DNA duplex by local pH changes combined with electric field effects were considered as a more plausible explanation.

Su et al.[342] used ITO-coated glass to study the rate of hybridization of ODN target sequences to DNA probes in the presence of an electric field. In absence of the electric field, hybridization required 10−30 h. Due to application of positive potential (200 mV) the hybridization rate dramatically increased, requiring only 10 min to 1 h. Although similar work was done previously on hybridization within a gel matrix,[293] Su et al. showed that deleterious EC reaction products expected by Sosnowski et al. could be prevented even when the probe was directly attached to the electrode surface.[342]

4.3. Oligonucleotides Covalently Bound to Electrode Surfaces

Intensive development of the DNA hybridization sensors stimulated further research into the DNA structure at the electrified surfaces. As mentioned above, in 1997 it was shown that electric fields can be used to regulate hybridization and denaturation of ODNs immobilized on solid electrodes.[293] Several authors have used optical surface plasmon resonance (SPR) to monitor hybridization kinetics in thiolated ODN monolayer on gold in presence of an applied electrostatic field.[343−345] In agreement with previous work,[293] the DC field either denatured surface-immobilized DNA duplexes or enhanced hybridization of DNA. Discrimination between matched and mismatched hybrids was achieved by proper adjustment of the electrode potential. The attractive DC field (+ 300 mV) was used to increase the rate of ODN hybridization. Application of the repulsive potential (−300 mV) to two-base-mismatched hybrids resulted in rapid denaturation of most of the immobilized duplexes (as detected by loss of ∼75% of target ODN) within a few minutes. On the other hand, fully complementary duplex DNA was detected after much longer exposure to the DC field. Compared to the DNA denaturation reported by Heller's group,[293] which immobilized their DNA in an agarose permeation layer, the monolayer DNA thiol films used by Georgiadis group[343,344] were attached directly to the gold surface. Immobilized DNA was thus exposed to a field gradient at the interface of the order of 10^9 V/m, similar to the DC field strength affecting DNA adsorbed at mercury[131,132] and ITO[342] electrode surfaces.

DNA hybridization assay based on electrochemiluminescence (ECL) detection was combined with surface potential controlling.[346] ECL is a term describing a reaction, which produces light at the surface of an electrode. Compared to fluorescence, ECL does not require an excitation, because the light generating reaction is triggered electrochemically. In addition, ECL shares characteristics of EC methods, such as highly localized reaction and spatial control. So far, only a relatively small number of ECL-based hybridization assays have been reported.[347−352] Gold electrodes were derivatized with 15-mer ODN probes conjugated with a synthesized ECL label.[346] Upon application of the potential of −300 mV for 150 s, the ECL signal of perfectly matched duplex remained unchanged, while the signal of the mismatched duplex decreased by ∼50%. Prolonged application of the negative potential decreased slightly the ECL signal of the fully matched duplex, while the signal from the mismatched one almost disappeared. These effects were observed in low ionic strength medium (30 mM phosphate, pH 7) but not at higher ionic strength (300 mM phosphate). These findings were in agreement with previous papers, as well as with theory.[353] According to Vainrub and Pettitt, electrostatic surface conditions influence stability of a formed DNA duplexes, and the distance up to which surface conditions influence surface bound species depends strongly on ionic strength of the solution.[353] Application of the negative potentials should more destabilize the mismatched duplex than the fully matched one, and the effect is expected to be more pronounced at lower ionic strengths.

4.3.1. Electrochemical Melting and SERS Monitoring.

Recently, Bartlett et al. used Surface Enhanced (Resonance) Raman Spectroscopy (SER(R)S) to follow denaturation of dsDNA attached to a structured gold surface (Figure 14).[354]

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This denaturation was driven either electrochemically (E-melting curves) or thermally on SERS active sphere segment void (SSV) gold substrates.[355] Using this method, it was possible to distinguish between wild type, a single point mutation, and a triple deletion in the CFTR gene at subattomole level (if only the DNA surface layer is considered). In addition to synthetic ODNs, PCR products of wt and mutant DNAs were used. The method was recommended as a new platform for detecting genetic variations in genes. Using a modified anthraquinone[356] in addition to Texas Red[354,357] as SERS markers, Bartlett's group further developed the conception of the negative potential-driven E-melting of the surface-immobilized DNA.[356,357] After improving discrimination between CFTR sequences of wt, 1653C/T (point mutation) and DF508 (triple deletion) in 22-mer ODN,[356] they attacked the problem of the analysis of short tandem repeats (STRs).[357] STRs are short repeating sequences of DNA (2−6 base pairs) which can be found at many loci within the human genome. Some of them are polymorphic and suitable for the identification of individuals as well as paternity/kinship testing, and disease-related linkage analysis.

To our knowledge, no EC methods of STRs detection have been published earlier, although Wakai et al. have suggested that the STRs could be, in principle, detectable by their chip developed originally for monitoring various single nucleotide polymorphisms.[358] Bartlett's group studied D16S539 polymorphic locus containing repetitive $(GATA)_n$, using asymmetric PCR with 5′-labeled primers to amplify ssDNA bearing a SERS label.[357] Purification of the PCR product was not necessary and a distinct SERS signal for the label was easily detected with no interference from other reagents in the PCR reaction mixture. As the potential was stepped more cathodic (in −100 mV increments) the spectral intensity of the label bands initially increased between −0.3 and −0.8 V and then decreased sharply as a result of the dsDNA surface melting. Such melting was accompanied by release of the labeled ssDNA from the duplex and its diffusion into the bulk of solution. The initial increase in intensity was reversible, involving probably a change in orientation of the label with respect to the SSV surface. Five different STR polymorphic sequences of D16S539 (10−14 repeats) were discriminated using the E-melting curves. These sequences ranged from 121 to 137 base pairs (bp) increasing in 4 bp increments, and were bound to a common capture probe (immobilized on the SSV substrate) containing 15 repeats of GATA and a 6 bp anchor region. EC melting potentials, E_m (value of the potential half-maximum), sensitively reflected differences in the lengths of the repetitive sequences, showing only 1 °C difference in their predicted thermal melting temperatures, t_m. The analysis required about 1500 molecules directly present under the laser spot and the analysis time was about 30 to 60 min.

4.3.2. Electric Field Effects. Wei et al.[69] discussed application of electric field in sensors for DNA diagnostics from different points of view. For example, they recommended application of the electric field not only during the recognition/hybridization but also before and after it. *Before the recognition*, the application of the electric field can help to control sample accumulation and separation,[359−362] control probe surface density[363] and arrange the probe molecules in a more uniform angle to the surface.[364] Dielectric forces can be generated for manipulating molecules within liquids,[365] etc. Numerous examples are available showing that application of positive potential *during the recognition* improved the speed and efficiency of the DNA hybridization.[344,359−364,366−371] For instance, detection of low-copy number of salivary mRNA without PCR amplification was reported.[371] In this work, Wei et al. utilized hairpin probe (see also section 5.5) in combination with specific binding of HRP system to the hairpin free end only after the hairpin probe transition to the duplex structure (resulting from the DNA hybridization). During the hybridization they applied cyclic square wave potential (30 cycles of +200 mV for 1 s and −300 mV for 9 s) to control the hybridization and HRP binding. Using 3′,3′,5′,5′-tetramethylbenzidine (TMB) as a substrate for HRP/H_2O_2 oxidation, they reported detection limit of ∼0.4 fM. Finally, *after recognition*, the nonspecifically adsorbed species can be removed away from the surface by means of applied electric field to obtain better signal-to-noise ratios (S/N). In any stage of the sensing process, alternating positive and negative potential pulses can serve for solution stirring.[368,371]

4.4. DNA Duplex Structure at Electrode Surfaces

In spite of a great progress in the development of the EC DNA hybridization sensors in the recent 15−18 years, our knowledge of the dsDNA structure at electrode surfaces is very limited as compared to detailed knowledge of DNA structure in crystals and in solutions.[308,372] It has been shown in thousands of experiments that ssDNA probes immobilized at surface hybridize with the complementary target ssDNAs forming duplex molecules. This duplex formation was analyzed by numerous methods, including EC and optical detection. While the duplex formation is without doubt, the question of the structure of the surface-immobilized DNA appears much less clear, because in-depth studies of this problem are missing. Moreover, structure of dsDNA lying flatly at the surface (e.g., at carbon electrodes) may differ from that of upright standing DNA molecule covalently attached to the surface via one of its ends. With ssDNA probe flatly laying at the electrode surface we cannot exclude the possibility that a ladder-like structure is formed as a result of its interaction with the cDNA strand diffusing from the bulk of solution.

It can be expected that the conformation of DNA molecules attached to the surface may significantly affect the efficiency of the DNA hybridization. Although a number of methods were applied (e.g., ellipsometry,[373] optical reflectivity,[374] neutron reflectivity,[375] SPR,[376,377] X-ray photoelectron spectroscopy,[378] and AFM[364,379−381]) to determine the structure of the DNA surface layer, they were not sufficiently sensitive to variations in the shape and structure of DNA molecules. Recently, the spectral self-interference fluorescence microscopy was applied to measure the average location of a fluorescent label in a DNA layer relative to the surface and to determine specific information on the conformation of the surface-bound DNA molecules.[382] Using this method, the shape of coiled ssDNA, the average tilt of dsDNA of different lengths, and the amount of hybridization were estimated. The measurements were, however, performed with DNA tethered to the glass surface and did not thus provide detailed information about the effect of different surface charges on the structure of the immobilized DNA. Spectroscopic ellipsometry in the infrared and vacuum UV range was used to study DNA (8−36 bases of ss and dsDNAs) covalently attached to diamond surfaces.[383] Average tilt angles of the DNA backbone with respect to the surface plane were determined ranging from 25° to 45° but no attempt was done to study effect of potential on the structure and properties of the immobilized DNA.

The conclusions resulting from the experimental work (see above) are supported by theoretical studies,[384] which suggest

that the melting temperature of dsODN is decreased by the repulsive negatively charged surface. In contrast, at positively charged surface the stability of the duplex is increased. These effects depend on the ionic strength and on distance of DNA from the electrode surface. At low ionic strength (e.g., in 10 mM NaCl) electrostatic effects are stronger because of the longer Debye screening length and the attractive surface may increase the DNA melting temperature to such an extent that DNA, which would not be able to hybridize in solution, may hybridize at the positively charged surface provided the DNA is covalently attached to the surface. In case of physically attached DNA, the ionic strength can affect not only the DNA duplex stability but also the adsorption strength. Further theoretical studies were performed to fill the lag between the fast development of DNA sensors and arrays on one hand and their physicochemical understanding.[385,386] Particularly, the mechanism of melting of DNA tethered to the a surface was studied in detail.[385] Conditions (temperature 400 K and 0.1 M NaCl) were chosen under which melting took place in microsecond time scale. On a silica surface DNA melting was dominated by fraying from the end away from the surface in difference to DNA melting in solution where both ends melted with roughly the same probability.

Generally, double-helical structure of DNA duplexes is expected in solution. Nevertheless, based upon modeling, flat nonhelical ribbon-like structures that may form under conditions of extreme mechanical distensions[340,387−390] or upon disruptive binding of an intercalator[391] were predicted. A ladder-like DNA duplex at the HMDE surface was considered by Nurnberg more than 30 years ago.[296] Recently, Lemeshko et al. concluded that on cationic surface at room temperature and usual conditions, the preferred duplex structure may not be a helix.[387] They immobilized 24-mer ssODNs by physical adsorption on a positively charged surface. The adsorbed ODNs formed densely packed monolayer, which retained its capacity for base-pair specific hybridization with complementary tDNA and duplex formation. On the ground of strand dissociation kinetics and the rate of DNase I digestion, it was concluded that on a positively charged surface a nonhelical DNA duplex can be the preferred structural isomer under standard biochemical conditions.

4.5. Concluding Remarks

DNA denaturation at negatively charged surfaces has been studied since 1974.[131,132] For >20 years this research was limited to mercury electrodes and only about 15 years ago the work with other surfaces was initiated.[189,293,392] Application of solid electrodes (which potential window is shifted by ~1 V to more positive values, as compared to Hg electrodes) resulted in (a) a better understanding of the stabilizing effect of positively charged electrodes on the immobilized DNA duplexes[293,344,392] and (b) confirmation of the destabilization/denaturation of duplex DNA at negatively charged surfaces. Beneficial effects of application of positive potentials on DNA immobilization and hybridization at carbon electrodes has been known already since 1996.[281,392,393] At mercury electrodes it was not possible to uncover the DNA duplex-stabilizing effects probably because of the mercury dissolution already at weakly positive potentials. Surprisingly, up to very recently,[354] the results of >20 years research of the DNA surface denaturation at Hg electrodes[131,132,231,289,294−296,299,300,326−329] were not considered by authors dealing with DNA surface denaturation at other electrodes.[293,343,344,394,395] We believe that this section will contribute to a better understanding of both the recent and earlier studies of the electric field effects on the surface-immobilized duplex DNA.

5. ADVANCES IN THE DEVELOPMENT OF DNA HYBRIDIZATION SENSORS

Up to 2001, EC sensing of DNA nucleotide sequences was based on DNA hybridization and EC detection on a single transducer/electrode surface,[187,393,396] in agreement with the definition stating that modern chemical sensor consists of physical *transducer* and chemically selective *material*.[397] On the other hand, other properties of the true chemical sensor, such as "continuous data acquisition", were neglected. In the Introduction to the Special Issue of *Chem. Rev.* on Modern Topics on Chemical Sensing,[398] J. Janata wrote: "There is some confusion in the terminology. Label 'chemical sensor' is often used to describe analytical procedure that should be correctly called 'analytical assay' or 'sensing system'. The main difference between the two lies in the mode of information acquisition. While true chemical sensor, for example, a smoke detector acquires information continuously, a sensing system, such an automated clinical blood analyzer obtains information in discrete steps. The two groups are fully complementary and valuable tools of analytical chemistry."

Considering strictly the above view and established criteria for the chemical sensor, practically all literature on sensing of DNA hybridization should be correctly called "sensing systems" or "analytical assays", but not DNA sensors. On the other hand, it appears hard to enforce the correct terminology because in thousands of papers on DNA sensing this terminology has not been followed. Specific properties of biosensing and particularly of DNA sensing may lead us to more relaxed definitions of biosensors claiming that biosensors are devices that combine[399] or integrate[400] a biochemical recognition element with a signal conversion unit (transducer). The latter definition[400] supposes that in NA-based biosensors, the NA has to be in an intimate contact with the electrode prior to and during the interaction with the analyte. In the following sections, we shall stick to this definition without discussing its correctness or incorrectness.

5.1. Single- and Double-Surface Techniques

In an attempt to improve DNA analysis in biological matrices, new technology was introduced in EC DNA sequence analysis about 10 years ago, which did not conform with the definition of true chemical sensor[397] and of the NA biosensor.[400] Instead of performing both the DNA hybridization and EC detection on the surface of the transducer (electrode) as in the so-called single-surface technique (SST), in double-surface technique (DST) the hybridization was carried out at one surface (such as paramagnetic beads, PMB, optimized for the given purpose) and EC detection at another surface, that is, the transducer. Optimum conditions for DNA hybridization (comprising recognition of the DNA complementary strands, Figure 2) greatly differ from those of EC detection of the hybridization event. For example, (a) the detection electrode (DE) should be small (capable to detect the smallest possible number of DNA duplexes resulting from the DNA hybridization). On the other hand, hybridization surface should be relatively large (to accommodate a large number of the probes for efficient capture of tDNA molecules from the sample); (b) nonspecific DNA adsorption during the DNA hybridization should be minimized, while adsorption of tDNA at the DE is beneficial in EC stripping analysis of tDNA (or of the reporter probe); (c) for

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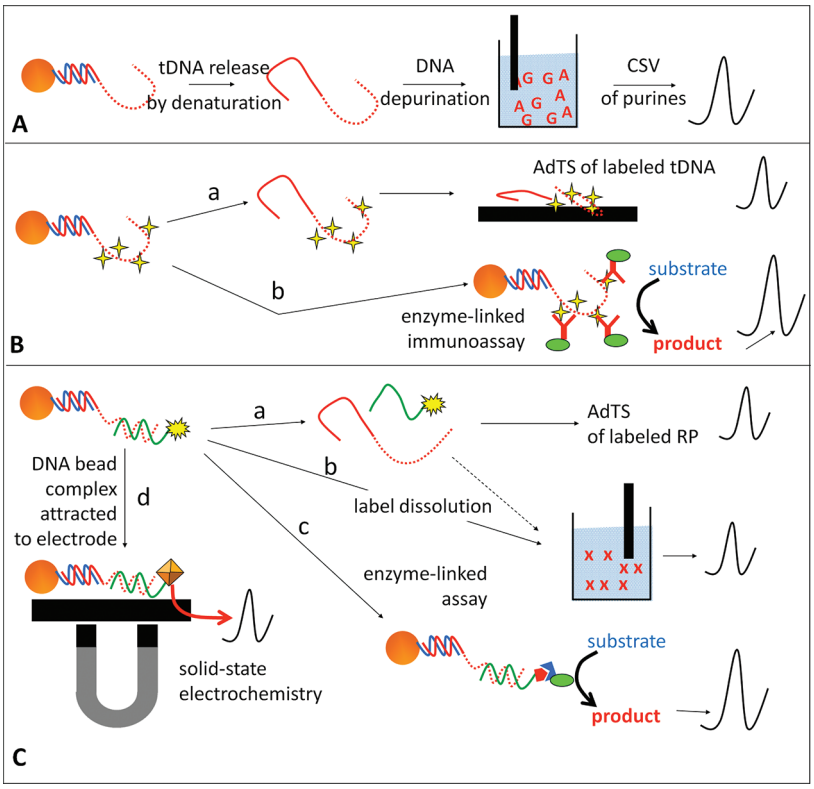

Figure 15. Some detection principles used in the paramagnetic beads (PMB)-based double-surface DNA hybridization technique. (A) Label-free detection of target DNA (tDNA). After hybridization of tDNA with a capture probe immobilized at the PMBs and magnetoseparation, the DNA is detached from the PMBs and depurinated with perchloric acid. Purine bases released from tDNA are determined using cathodic stripping voltammetry (CSV) at HMDE at subnanomolar[175] concentrations and at solid amalgam[175] or carbon electrodes[180] at nanomolar level. (B) End-labeling of DNA. Electroactive labels such as osmium tetroxide complexes (Os,L) are covalently attached to tDNA or to the reporter probe (RP). After hybridization, (a) the electroactive tags are determined, for example, by ex situ adsorptive stripping voltammetry, or (b) EC enzyme-linked immunoassay can be used for detection of labeled DNA, if antibody against the label is available.[422] (C) "Sandwich" assays. RP tags can be detected using (a) the ex situ stripping (e.g., for Os,bipy-labeled RPs), (b) nanoparticle-based DNA tracers or "electroactive beads" determined after their dissolution,[423] or enzyme-linked assays with biotinylated DNA and streptavidin-enzyme conjugates. Metal nanoparticles as well as metal sulfide nanocrystals can be detected via solid-state EC measurements after magnetic attraction of the entire PMB-tDNA-RP assembly to the electrode. Reprinted with permission from ref 406. Copyright 2007 Elsevier.

the DNA hybridization the DE should be interfaced to decrease the noise. On the other hand, interfacing of DE may decrease the measured DNA signal, etc. In June 2001 at the XVI. Bioelectrochemistry Symposium in Bratislava, J. Wang and one of us (E.P.) independently reported on the new DST for EC sensing of the DNA hybridization.[21,401,402] Major part of DST development was recently reviewed,[403−406] here only a brief summary will be given.

Due to minimizing of nonspecific DNA adsorption in DST, very high specificity of the DNA hybridization was reached. Using commercially available PMBs, both label-free[175,248,407−409] and label-based[250,410−413] EC detection of DNA with DST were reported. J. Wang mastered the DST by combining it with metal and semiconductor nanoparticles, nanowires, electroactive beads, CNTs etc., reaching the highest sensitivity of the DNA detection in EC sensors in the middle of the 2010s[414−418] (see section 5.3 for details).

In DST, optimum DE, as well as the EC technique, can be chosen with respect to the given electrode process, without considering the conditions for the hybridization. High sensitivity of stripping techniques, based on accumulation of DNA or its label at the DE, can be thus utilized (Figure 15). In a variety of protocols the hybridized NAs were (a) either detached from the PMB, followed by their stripping detection[175,401,408] or (b) the PMB-NA complex was (i) magnetically transferred to the electrode surface[409,414] or (ii) the PMB-NA-enzyme "sandwich" was transferred into a substrate solution, followed by EC detection of the consumption of the electroactive substrate or of formation of the enzymatic reaction product.[419,420] In the first decade of this century, DST played an important role because of very good protecting of hybridization surface (represented mostly by commercially available PMB) from the nonspecific adsorption. Recent advance in interfacing of gold electrodes with binary and ternary thiol SAM's suggested that the single-surface techniques (SST) can be made competitive to or even surpass DST (using the well-established PMB) in their efficiency in prevention of nonspecific adsorption of NAs and proteins (see section 6.3.2 for details).[421]

5.2. Label-Free and Label-Based DNA Sensing

Label-free and label-based techniques have been used in both DST and SST approaches. Label-free methods are simpler but for several years they rarely reached sensitivities comparable to the label-based methods. With the recent arrival of improved shielding of the electrode surface and combination of enzymatic and chemical catalysis, the label-based SST methods are getting a chance to surpass DST in sensitivity and specificity.

5.2.1. Label-Free Techniques. Label-free techniques are usually based either on intrinsic electroactivity of target NA molecules, or on detection of physical changes associated with the hybridization reaction, such as changes in resistance or conductivity of an interface modified with duplex DNA as compared to ssDNA. Most common strategies used in label-free sensors are depicted in Figure 16. For example, intrinsic

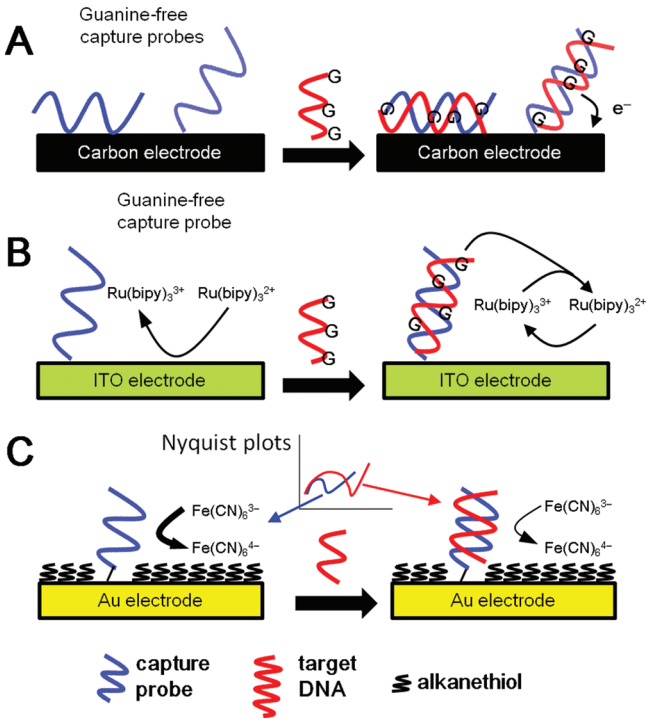

Figure 16. Overview of label-free hybridization schemes commonly used in EC DNA hybridization sensors. (A) Direct oxidation of target DNA guanine bases after the hybridization with inosine-modified capture probe (i.e., devoid of guanine). (B) Electrocatalytic oxidation of guanine by means of redox mediator. (C) Increase in electron transfer resistance between electroactive redox couple and the electrode surface due to a formation of the duplex, as measured by EC impedance spectroscopy. Other electrodes were also applied for label-free hybridization sensors, for example, mercury and solid amalgam electrodes. (A) Adapted with permission from ref 434. Copyright 2009 American Chemical Society. (B) Adapted with permission from ref 429. Copyright 2000 American Chemical Society. (C) Adapted with permission from ref 435. Copyright 2009 Elsevier.

signals of NAs can be obtained through an oxidation of G residues in tDNA molecule. The capture probe must be devoid of G (and thus the tDNA should not contain any C), otherwise G's in the probe would contribute to the background signal. This problem can be circumvented if the G present in the capture probe is substituted with structurally similar inosine (capable of binding to the C as well) (Figure 16A).[196,424−427] Inosine (I) is oxidized at different potential and thus any signal from the oxidation of G can be assigned to the tDNA (the resulting duplex containing I•C pairs is however less stable than that with G•C pairs). The method was elaborated only for ODNs of short lengths. If the tDNA is hundreds of nucleotides long (or even longer), and almost all G residues are involved in the electrode process, then relatively small number of G residues in the shorter probe DNA can be neglected. This is the case when DST is used, followed by DNA depurination and determination of free G and A residues by stripping techniques (Figure 15A and section 3.1.3).[175,248] In this method all purine residues contribute to the signal. In such a case, sensitivity of the determination increases with the length of tDNA. If the tDNA is not much longer than the probe DNA, tDNA can be easily separated from the probe strongly bound to the surface. On the other hand, if the probe lies flatly and is electrostatically bound to the carbon electrode, only a small portion of G residues of long tDNA are in contact with the electrode, while the rest remain in the solution without contributing to the signal. In such a case sensitivity of the determination cannot increase with the length of tDNA.

G oxidation signals can be amplified when coupled to the electrocatalytic cycle in the presence of a redox mediator, for example, $[Ru(bipy)_3]^{2+}$ (Figure 16B).[170,428−433] After the hybridization step, Ru(III) previously generated by applying an oxidation potential, is regenerated back to Ru(II) by a catalytic oxidation of G present in the tDNA. Despite the use of the mediator, such technique can be still considered as label-free (although not reagentless).

5.2.2. Electrochemical Impedance Spectroscopy. In recent years, EIS has been increasingly applied in the DNA hybridization and sensors.[37−40,268,436] EIS allows analyses of both resistive and capacitive properties of electrode-attached materials, including DNA, RNA, and proteins. Such analyses are based on perturbation of a system at equilibrium by small amplitude sinusoidal excitation signals.[268,437] It is advantageous that impedance of the system can be scanned over a wide range of AC frequencies. Predominatly, frequencies in the range between 100 kHz and 10 MHz have been used to obtain impedance spectra controlled predominantly by the interfacial properties of the modified electrodes. Theory of EIS was described in detail in several reviews.[39,40,268,436]

EIS is frequently applied for probing the electron transfer resistance, R_{et}, of the NA-electrode surface. Any electrode modifier having insulating features tends to increase the electron transfer resistance - a phenomenon widely used in impedimetric sensors.[268] In its simplest form, no additional reagents are required, and only change in R_{et} after dsDNA attachment is monitored.[438] More common strategy is to use an indicator, e.g., ferricyanide/ferrocyanide redox couple, to test the R_{et} after the hybridization with tDNA (Figure 16C).[435,439] Amplification is, however, needed to obtain higher sensitivity. This is usually achieved with various surface modifications[440−443] or by attaching additional "layer-forming" labels to DNA, such as nanoparticles,[444] quantum dots,[445] or liposomes,[446,447] having a considerable effect on resulting impedance. However, the approach with additional layers cannot be strictly considered as label-free. EIS method is based on an assumption that no impurities capable to form an insulating layer are present in the DNA sample. This assumption can be easily fulfilled when synthetic ODNs are used. Difficulties may, however, arise if biologically relevant NA samples are analyzed (section 6).

5.2.3. DNA Labeling and Label-Based Techniques. Main reason for application of external electroactive species is to increase sensitivity of DNA determination, outweighing the drawbacks they bring about - higher complexity, price, and more laborious and time-consuming work; other advantages include application of less extreme potentials for oxidation/reduction of the label (as compared to DNA bases) or its reversibility. At first, simple intercalators or groove binders (binding preferentially to dsDNA) were used as redox indicators in DNA hybridization sensors, but later it was

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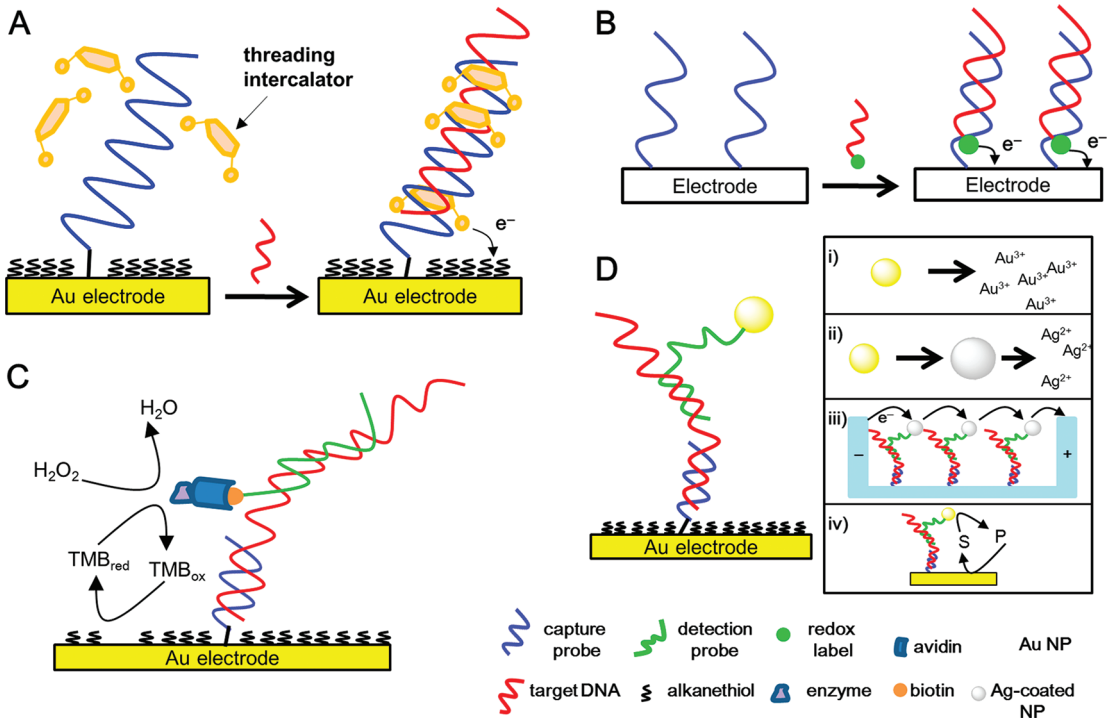

Figure 17. Several label-based hybridization schemes applied in EC DNA hybridization sensors. (A) Detection of duplex formation using a threading intercalator, intercalating strongly but noncovalently into dsDNA. (B) Detection method based on covalent labeling of the target DNA with an electroactive molecule. (C) A sandwich technique using two complementary probes, capture probe immobilized at the solid surface and labeled reporter probe (e.g., with biotin, antigen, etc.), to which avidin-enzyme (or antibody-enzyme) conjugate is bound. Enzyme catalyzes reaction, which product can be electrochemically detected; the oxidized (or reduced) product can be then regenerated using a mediator. In some cases, it is the peroxidase/H_2O_2 substrate (such as TMB), which is electrochemically detected. (D) Nanoparticle-based sandwich technique with multiple detection schemes: (i) dissolution of Au NP followed by detection of Au^{3+}; (ii) signal enhancement with silver ion precipitation on the surface of the NP; (iii) conductometric approach using silver deposition facilitated by the gap-bridging NPs, leading to measurable conductivity changes; (iv) utilization of catalytic properties of the NPs. S, substrate; P, enzymatic product; NP, nanoparticle. Note that the strategies depicted in (C) and (D) are not dependent on a distance between the electroactive species and the electrode, since low MW molecules are generated which diffuse to the surface. In (A-B), the position of the label affects the resulting signal. (A) Adapted with permission from ref 452. Copyright 2005 American Chemical Society. (C) Adapted with permission from ref 461. Copyright 2006 American Society for Microbiology.

shown that they did not possess sufficient discriminating ability for dsDNA. Some binding, although to much lower extent, occurred also with ssDNA due to an electrostatic attraction (many intercalators were positively charged), and also due to interaction of the intercalator with sequence-dependent short helical regions in longer tDNA, resulting in lower S/N ratio. Recently, Gebala et al. introduced a proflavin intercalator, which they believe is more specific toward dsDNA.[448] The proflavin intercalator had an advantage that it could be functionalized with two biotin molecules by postlabeling method (i.e., after the intercalation into the dsDNA).[25] Such biotinylated proflavin interacted with the streptavidin, conjugated with an enzyme, which amplified the resulting signal. A higher affinity toward dsDNA was displayed by complex intercalators, such as bis-intercalators echinomycin[22,23] and naphthyl imide functionalized diviologen,[449] or naphthalene diimide-based threading intercalators (Figure 17A).[24,450−454]

Another approach is based on an electrostatic interaction between negatively charged DNA sugar−phosphate backbone and a redox indicator, which is either positively or negatively charged. Positively charged indicator (e.g., $[Ru(NH_3)_6]^{3+}$) is attracted to the DNA backbone, and higher ruthenium signal is obtained after the formation of the duplex (due to an increased negative charge density of the duplex).[455,456] However, such design inherently suffers from high background current due to negative charge of the probe DNA. An interesting solution to circumvent the problem was to use a PNA probe, having a pseudopeptide backbone without an electric charge (see section 3.3.1).[392,457,458] In this way, cationic species (usually transition metal complex, such as $[Ru(NH_3)_6]^{3+}$) did not interact electrostatically with the PNA probe, and thus the signal could be attributed to hybridization with negatively charged tDNA.

In the case of a negatively charged indicator, repulsion between a negatively charged DNA backbone and the indicator, such as $[Fe(CN)_6]^{3-}$, is usually manifested by a decrease in the current. For instance, using scanning electrochemical microscopy (SECM) significant decrease in positive feedback currents was observed above spots of duplex DNAs. The SECM tip, acting as an ultramicroelectrode, was kept at sufficiently negative potentials to reduce $[Fe(CN)_6]^{3-}$, and thus a presence of the duplex under the tip led to the decrease of the reduction current.[459,460]

In difference to free-diffusing electroactive molecules (redox-active mediators, intercalators, groove binders, etc.), covalent labels provide higher stability and reliability due to well-defined bonding. Electroactive labels are covalently attached to either target NA (Figure 17B) or to reporter probe used in a sandwich assays (Figure 17C, D). The latter approach involves immobilization of the capture probe onto the solid surface (electrode, magnetic beads, etc.), followed by hybridization with the target NA. After the first hybridization step, labeled reporter probe with a sequence complementary to the different

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part of the target NA is introduced (Figure 17C). Examples of labels covalently attached to NAs include osmium tetroxide complexes with nitrogenous ligands [Os(VIII)L; or simply Os,L],[250,410−412,462−471] ferrocene derivatives,[472−481] ruthenium complexes,[482] anthraquinone,[356] aminophenyl and nitrophenyl groups,[483,484] polyhedral boron clusters,[485] enzyme tags,[420,422,465,486−497] nanoparticles,[60,423,498−508] or quantum dots,[509,510] CNTs,[413,511] etc.

Both probe and target NA strands can be labeled. To our knowledge, the first electroactive markers covalently bound to the DNA were Os,L, introduced by our group already in the first half of 1980s[126−128] and later they were used for labeling of target/reporter probe DNAs[21,411,464,512] Os,L complexes react preferably with pyrimidine moieties (T ≫ C) in single-stranded, but not in intact dsDNA molecules (Figure 18),

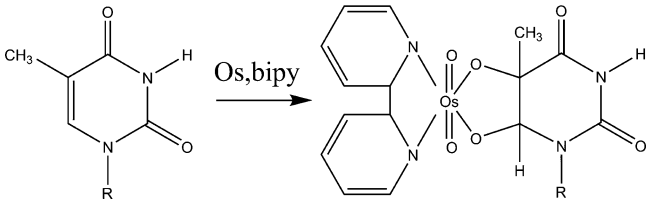

Figure 18. Reaction of Os,bipy with thymine in single-stranded DNA form (Os,bipy = complex of osmium tetroxide and 2,2′-bipyridine).

enabling thus discrimination between these two DNA forms.[308] High sensitivity of some Os,L complexes for changes in DNA structure was utilized in DNA structure analysis and particularly in studies in local DNA structures stabilized by supercoiling in vitro[308,513] and in cells[308,514,515] by means of gel electrophoresis, but not by EC analysis. Bhattacharyya and Lilley[516] showed that mispaired T bases are reactive to Os,L, and C bases to hydroxylamine. Using Os,bipy, their finding was recently utilized in EC detection of abasic sites, as well as of mispaired (e.g., G·T pairs) and unpaired (inserted/bulged) T bases in DNA duplexes at carbon electrodes.[465,517] Analysis of changes in DNA duplex structure based on combination of chemical probes of the DNA structure with EC detection appears simple and reliable and deserves further attention.

End-labeled DNA-, RNA-, or PNA-Os,L were detected voltammetrically at carbon,[410,412,462−465] gold,[466−469] or mercury electrodes.[250,411,464,470,471] The most studied complex was perhaps Os,2,2′-bipyridine (Os,bipy), producing redox couples at carbon, gold and mercury electrodes. At carbon electrodes, separation of the modified electrode from the reagent solution was not necessary if AdTS technique was used.[462] Whole reaction mixture was dropped at the surface and weakly adsorbed, unreacted Os,bipy was removed during washing, while the Os,bipy-DNA adduct remained firmly adsorbed at the electrode. It was also shown that DNA adducts obtained with Os complexes containing different ligands produced redox signals with different peak potentials (E_p), utilized in "multicolor" DNA labeling.[412] At gold electrodes, multiple reduction of Os was analyzed voltammetrically after hybridization of a thiolated capture probe with Os,L-modified ODN target.[466−469,518−521] Mercury electrodes in combination with Os,bipy produced not only redox couples, but also much more sensitive catalytic peak at ∼−1.2 V, providing means to determine NA concentrations below ng/mL ODN.[464,471]

Alternatively, a capture probe can be modified and immobilized at the electrode surface. For instance, Fc is frequently placed at the flexible end of the oligonucleotide capture probe, which prior hybridization adopts a conformation in which Fc moiety is in the vicinity of the electrode. This enables an efficient electron transfer to proceed until the hybridization occurs, resulting in duplex formation and adoption of upright conformation, moving away Fc from the electrode and decreasing the current. More details regarding these types of sensors based on their conformational changes can be found in section 5.5. Recently, it was shown that methylene blue (MB) as a covalent label exhibited better long-term storage and reproducibility, as compared to Fc. Sensors with MBs were superior when employed into complex sample matrices, such as blood serum.[479]

Recently, an interesting approach was developed to modify deoxynucleoside triphosphates (dNTPs) with various electroactive functional groups, using simple aqueous phase cross-coupling reactions,[30] and incorporate them enzymatically into DNA by polymerases either by primer extension or by PCR.[31] The successful incorporation of Fc,[481,522−525] anthraquinone,[523,524] biotin,[525] amino- and nitrophenyl,[483,484] or Ru/Os(bipy)$_3$ tags[482] for EC detection, or for attachment of reactive aldehyde groups for further modification,[526] was demonstrated. Moreover, after the incorporation of dNTPs, DNA could be further modified by restriction endonucleases.[527,528] A systematic work with 13 different restriction endonucleases showed that an introduction of certain modifications into the recognition sequence might be useful for protection of the sequence from cleavage by the enzymes, while the modifications localized next to the recognition sequence did not, in most cases, inhibit the cleavage. Similar approaches based on enzymatic incorporation of labeled nucleotides were also applied using dNTPs, bearing affinity tags such as biotin, to create DNA targets ready for attachment of an enzyme-streptavidine conjugate followed by enzyme-linked EC detection[493,529] (for more details see below). Biological applications involved e.g., (a) SNP detection using a set of dNTPs bearing different electroactive or affinity/enzyme labels,[482,484,529] (b) hybridization experiments with target sequences derived from p53 gene,[483] (c) monitoring of gene expression in real biological samples (section 6),[493,529] or (d) differentiation between specific and nonspecific interactions of p53 protein to DNA binding site.[483] Protein-binding experiments were performed using PMBs covered with protein G (which bind to a highly conserved region of antibodies) and a p53 antibody. Antibody-p53-DNA complex was thus attached to the PMB, washed, and labeled DNA was dissociated and analyzed voltammetrically.

Sensitive determination of NAs can be also achieved with the use of enzymes,[403] which are usually bound to NA via one or more linkers. Mostly, the enzyme is conjugated to another protein, for example, antibody or (strept)avidin, strongly binding an antigen- or biotin-labeled oligonucleotide, respectively. Enzymes catalyze a conversion of added substrate into the product, which is then electrochemically determined. Very low detection limits are brought about by conversion of multiple substrate molecules per single enzyme (and thus per single DNA duplex formed). Basically two strategies can be distinguished. In the first one, labeling of the target with biotin or antigen (FITC, Os,bipy, etc.) is performed, followed by addition of avidin- or antibody-enzyme conjugate. Only two complementary strands (capture probe and target) are required. Labeling is relatively easy if the target molecule is modeled by short ODNs present in simple solution. However, labeling of the real target NAs in complex media might be difficult.

Thus a second strategy, a sandwich assay, was developed, in which three DNA strands are used; two of them (capture and reporter probes) are complementary to two different regions of the target DNA strand (Figure 17C). In the sandwich assay, the capture probe is first immobilized at the electrode surface, followed by capturing the target DNA (hybridizing with the capture probe) and addition of the reporter probe, hybridizing to the complementary sequence in the target ssDNA. Products of enzymes such as horseradish peroxidase (HRP), alkaline phosphatase or bilirubin oxidase were determined with various EC techniques, including amperometry,[486−490,530] EIS,[491] voltammetry,[422,465,492−495,529] or potentiometry.[420,496,497] Sensitivity of the sandwich assays was greatly enhanced by using TMB as a substrate for HRP/H_2O_2 oxidation.[531] The enzymatic product is electroactive, producing cathodic and anodic peaks at −0.04 and +0.14 V (against Ag/AgCl electrode) at pH 4.0.[532,533] Systems involving this type of sandwich assays in combination with efficient screening of Au electrodes appear particularly useful in the analysis of biologically relevant NA samples without PCR amplification (section 6).

5.3. Nanotechnology in DNA Sensors

Implementation of nanotechnology represents a powerful alternative to common strategies for creating smaller-sized, more rapid and less expensive EC devices for tDNA recognition. By applying various nanomaterials acting either as nanoelectrodes, immobilization substrates for accumulation of increased amounts of DNA probes, or as signal amplifiers of the hybridization event itself, higher sensitivity of tDNA detection has been achieved. Due to excellent electrical, mechanical or catalytic properties of the nanomaterials, they have been subject of many papers, accompanied by plentiful reviews[55,58,60−63,65−67,69−71,74,79] in the last three years.

5.3.1. Nanoelectrodes. In addition to the signal enhancement, nanoelectrodes can provide an extra benefit in terms of device miniaturization and portability, lower consumption of the sample, or construction of high density sensor arrays.[61] However, Poissonian sampling errors may arise when analyzing small volumes of low-concentration analytes, because such volume might not be representative of the true sample concentration.[534] Lost of kinetic advantages due to a close electrode spacing,[535] or reproducibility of nanoelectrode fabrication also remain a concern. Semiconductor or metal nanowire electrodes functionalized with DNA probes were successfully applied in the detection of DNA hybridization.[536−541]

5.3.2. Nanoparticles. Variety of nanoparticles (NPs), including noble metal, metal oxides, polymeric or semiconducting NPs were applied in order to improve a detection of hybridization event. Frequently used metal NPs (e.g., gold, platinum, palladium, etc.) provide several advantages,[542] such as (1) increase of the surface area upon their immobilization on the transducer surface, leading to more sensitive EC detection of NAs and other biomolecules,[498,499] (2) remarkable conductivity,[500,501] (3) adjustability of the properties by controlling their size or morphology,[366,416,510] or (4) signal amplification of the hybridization reaction.[60,423,502−508] For example, the amplification can be achieved by exploiting catalytic properties of the metal NPs,[502,503] or by oxidative dissolution of the metal NPs to metal cations, usually followed by stripping voltammetric or potentiometric detection.[60,423,504,505] Some studies went further and used precipitation of silver ions at the surface of the gold NP and their subsequent "stripping".[506−508]

Semiconducting NPs, having size-tunable properties, and metal oxide nanoparticles, with their high mechanical and thermal stability, as well as negligible swelling in solutions, represent another promising material for DNA hybridization detection. Arsenal of such NPs include metal sulfides (e.g., CdS, ZnS, PbS, CuS) acting as electroactive labels[509,543−545] which offer multiplexed capability for simultaneous tDNA detection (Figure 19),[510] ZrO_2,[546] ZnO,[547] CeO_2,[548] or magnetic iron

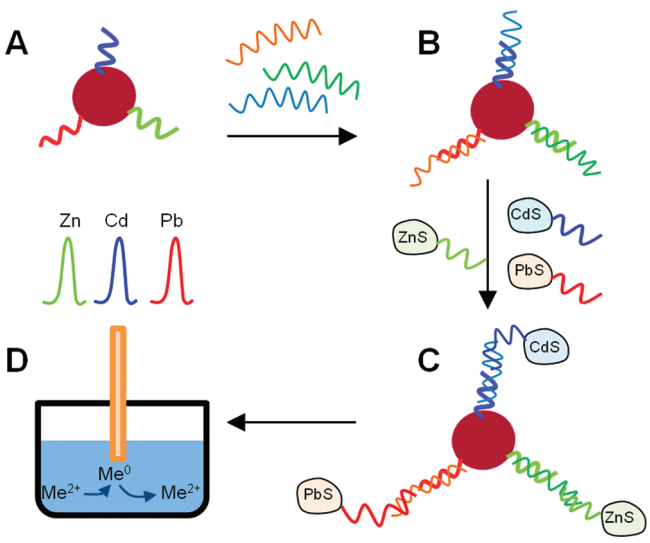

Figure 19. Multitarget electrical DNA detection protocol based on different inorganic colloid nanocrystal tracers. (A) Introduction of capture probe-modified magnetic beads. (B) Hybridization with the DNA targets. (C) Second hybridization with the quantum dot-labeled reporter probes. (D) Dissolution of quantum dots and EC detection. Adapted with permission from ref 510. Copyright 2003 American Chemical Society.

oxide nanoparticles[549,550] (do not interexchange with commonly used magnetic microparticles, discussed in section 5.1), serving as substrates for DNA immobilization.

5.3.3. Carbon Nanotubes. Combining the advantageous properties of traditional carbon-based electrodes with those of nanomaterials, CNTs display enhanced electron transfer, catalytic behavior, good biocompatibility, high mechanical resistance, rapid electrode kinetics, and high surface-to-volume ratio for increased accumulation of biomolecules.[74] Usually two distinct structural families of carbon nanotubes are distinguished, single-wall carbon nanotubes[551] and multiwall carbon nanotubes, consisting of concentric and closed tubules.[552] Electrocatalytic effect of CNTs attributed to the edge-plane sites at the ends of the CNT, similar to the edge-plane pyrolytic graphite electrodes,[553,554] was further improved by introducing bamboo-like CNTs with higher number of edge planes.[555] DNA can be attached to the CNT surface either directly[213,219,556] or in connection with various polymers[557,558] and NPs.[559−561]

When using a label-free design, CNT's attachment to the electrode surface leads to a large enhancement of intrinsic G oxidation signal,[213−219] which can be further enhanced when coupled to $[Ru(bipy)_3]^{2+}$, completing thus an electrocatalytic cycle.[562] Additional improvements of detection limit could be obtained in a label-based strategy developed by Wang's group.[413,511] CNTs played dual role here, namely as carriers of numerous enzyme tags and for accumulating the product of

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the enzymatic reaction, enabling detection of subpicomolar ODN concentrations. More detailed information regarding general properties of CNTs and particularly their use in EC sensors can be found in recent reviews.[71,72,74,78,79,82,563,564]

5.4. Electronically Conducting Polymers

Electronically conducting polymers (ECPs) belong to a group of polyconjugated unsaturated polymers with properties of both metals and organic polymers. Compared to saturated polymers, ECPs have a unique electronic structure, responsible for their electrical conductivity, low ionization potentials and high electron affinity.[265] Because of their porosity, ECPs are easily penetrated by gases capable of changing their electronic properties.[565] This property of ECPs has been utilized in a number of gas sensors.[565−567] ECPs are used not only as the selective layer in sensors, but also as the transducer itself,[565] being sensitive to biorecognition events affecting their interfacial properties, including DNA hybridization. These events bring about changes in ECP properties such as conductivity, making them suitable for sensor applications. In their ground state, ECPs exhibit semiconducting or even insulating properties, but the conductivity can be obtained by doping, that is, by oxidation (p-doping) or reduction (n-doping) of the π-electronic system.[566]

ECPs can be polymerized both chemically[568] and electrochemically.[569,570] The latter one, termed electropolymerization, is advantageous for DNA sensing, because (1) the polymer film is confined to the electrode, determining its shape; (2) polymerization is performed at ambient temperatures and (3) properties of the ECP films, such as thickness, can be adjusted by varying EC polymerization conditions. Two most common approaches for EC polymerization involve potentiostatic (potential-controlled) and galvanostatic (current-controlled) polymerization.[571] More information on electropolymerization of various ECPs can be found in recent review.[569]

Perhaps the most widely used ECPs in DNA sensors are polyaniline,[572−576] polypyrrole,[577−584] and polythiophene.[585−587] Polyanilines, which use in biosensors was recently reviewed,[572] exhibit several tunable properties, including conductivity, color transition or porosity. In addition, their thickness can be controlled and are environmentally stable. Polypyrroles manifest strong absorptive properties toward biomacromolecules, biocompatibility and they can be easily deposited at the electrode surface. Chemical structures of these widely used ECPs are illustrated in Figure 20.

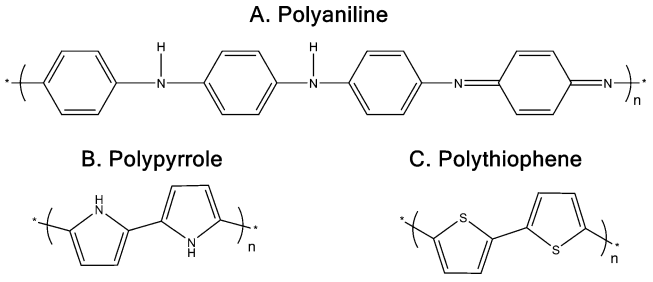

Figure 20. Chemical structures of electronically conducting polymers most widely used in DNA biosensors.

Different ways of DNA immobilization to ECPs are possible, and all of them are aimed at retaining ODN's probe ability to efficiently hybridize with target NA molecule. The main strategies involve (a) entrapment within the polymer matrix during its electrochemical growth, (b) covalent bonding between the DNA and functionalized polymers, and (c) affinity interactions-based attachment of the DNA. Entrapment in electropolymerized films, applicable to a variety of biomolecules, consists of a physical incorporation of the biomolecule (found in the vicinity of the electrode) into the growing polymer. The advantage is that this approach is reagentless and occurs under mild conditions, keeping the biomolecules in active state. Problems can arise due to a steric hindrance or when the polymer is incompatible with the entrapped biomolecule. Moreover, the approach requires higher starting amount of monomers and biomolecules. Wang et al. first reported that ODNs could act as sole charge-compensating counterions during the growth of polypyrrole films, while keeping the ODNs ready for hybridization;[588,589] other papers soon followed.[579,590] Covalent attachment of ODNs to ECPs may provide a better access of the target NA molecules to hybridize with the ODN probes. ODNs are usually functionalized with reactive groups (e.g., $-NH_2$, $-COOH$), followed by an attachment to functionalized monomers for copolymerization, or to already formed polymer film. Copolymerization of pyrrole monomers with pyrrole-bearing ODNs for development of electrode arrays was demonstrated.[591,592] On the other hand, the electropolymerization of functionalized conducting polymers can be performed first, followed by the attachment of biomolecules to the polymer film by chemical grafting. Benefit of this approach is that the conditions for each step can be optimized.[266,442,593,594] Further improvement in terms of a probe orientation was achieved with affinity interactions-based method. For instance, avidin−biotin interaction was used, in which biotin-functionalized ECPs were coupled with avidin molecules, followed by introducing biotinylated ODNs.[574,581,595]

Similarly to DNA hybridization assays not involving ECPs, both label-free[579,596] and label-based[573,575,578] approaches are distinguished. For instance, a new label-free DNA hybridization assay based on the exchange of chloride ions between the polypyrrole layer and the buffer was developed.[583,597] Addition of a negative charge to the electrode surface, represented by a complementary target ODN, hindered the chloride ion exchange, detectable as a decrease of CV current. The strategy was recently used to create an ODN microelectrode array.[582]

In both of above-mentioned approaches, ECPs played merely a passive role, acting as a DNA immobilization substrate and electronic relay. However, ECPs can also directly affect transduction process, manifested as a change in ECP redox behavior, conductivity, etc.[442,577,580,593,598] To achieve this, ECPs, mostly cationic polythiophenes, have been functionalized with an electroactive moiety, such as ferrocene.[585,599] Ferrocene-functionalized polythiophenes did not serve as immobilization substrates for DNA probes, but rather acted as a label of the hybridization event. For this purpose, neutral PNA probes were attached to the electrode surface and when complementary, negatively charged tDNA hybridized with the probe, cationic polythiophene was introduced, interacting electrostatically with the resulting complex. Ferrocene moiety served as an electroactive species undergoing redox processes, allowing determination of picomolar concentrations of tDNA.[599]

Naturally, it is not feasible to select one single ECP that could be generally applied for wide use in DNA biosensors; each possesses advantages but also limitations. Until now, most studies involving ECPs-based DNA detection were conducted

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with short synthetic ODNs;[573,574,577,578,600,601] application of real NA samples is still rather low (summarized in Table 3).

Table 3. Several Examples of ECP-Based DNA Detection Assays Using Real NA Samples[a]

polymer	target NA	electrode/ support	ref
polyaniline	miRNA	Au/Ti microelectrodes	606
polyaniline	mRNA-transcribed PCR-amplified DNA of BRCA1,[b] H4 and GAPDH genes	Au	607
polypyrrole	hepatitis C virus RT-PCR-amplified RNA from serum	Au microelectrodes	592
polypyrrole	PCR-amplified DNA from bovine leukemia virus-infected cells	Pt	579
polypyrrole	microbial rRNAs from *E. coli*	Pt	582
polythiophene	plasmid-cloned Mariner transposons	Pt	587

[a]More about EC analysis of real samples detection can be found in section 6. [b]BRCA1, breast cancer gene 1; H4, histone H4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription PCR (see section 6.1.1).

However, it is indisputable that ECPs will continue to play an important role in development of new EC DNA assays. More information on ECPs can be found in reviews focusing on their general use in chemical and biological sensors,[566,602,603] application to gas sensors,[565] DNA sensors,[265,604,605] electropolymerization,[569,570] and use of polyanilines,[572] or polypyrroles.[266,597]

5.5. Electrochemical Molecular Beacons

In the process of DNA renaturation or hybridization in solution, complementary ssDNA molecules bind to each other forming the well-known DNA double helix. Some properties of the two DNA strands in the double helix greatly differ from those of free ssDNA molecules. For example, in ssDNA bases are freely accessible while in the double helix they are hidden in the interior of the molecule, involved in hydrogen bonding and stacking.[27,308] Accessibility/inaccessibility of bases strikingly influence chemical reactivity, adsorbability and redox processes at electrodes (Figures 4 and 6), as well as biological activity of DNAs. These differences in properties of ds and ssDNAs, primarily observed at Hg electrodes, were utilized for various purposes, including EC tracing of the DNA denaturation and renaturation.[104,123] Other differences can be mentioned, such as flexibility of DNA single strands and rigidity of the rod-like DNA duplexes, etc.[26,27,308] Moreover, DNA forms local structures, frequently stabilized by DNA supercoiling,[308] such as cruciforms, hairpins, triplexes, etc., in which duplexes are combined with ss regions; such local structures, as well as unnatural structures can be modeled by ODNs.[372,608] Differences in DNA flexibility of ss- and dsDNAs and structural transitions in hairpins (stem-loop structures) have been utilized in the development of a new type of the electrochemical DNA sensor (E-DNA sensor)[609] which has been significantly improved in recent years.[84,85]

About 8 years ago, Fan et al.[609] invented first-generation E-DNA sensor in which one end of the DNA hairpin was immobilized on a gold electrode via the terminal −SH group while the other end carried a ferrocene label, integrating thus functions of the capture probe and the reporter probe in a single hairpin architecture (Figure 21). Analogous to fluorescent molecular beacons,[50,610−612] E-DNA sensors rely

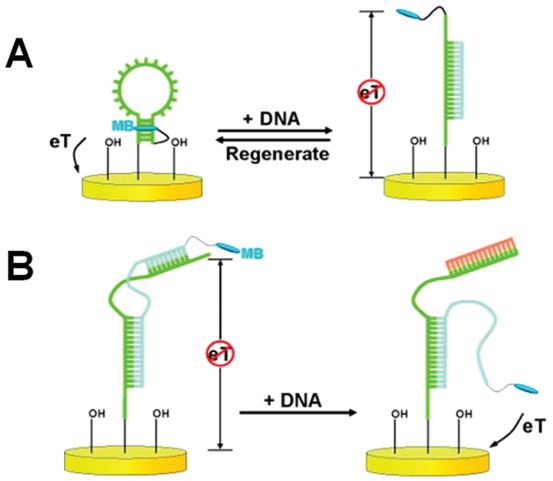

Figure 21. Scheme of the E-DNA sensor based on target binding-induced conformational changes in DNA structure. (A) "Signal-off" E-DNA architecture, comprising a redox-tagged stem-loop DNA attached to a working electrode. In the absence of target, the redox tag is held in proximity to the electrode, ensuring efficient electron transfer (eT) and a large, readily detectable faradaic current. Upon hybridization with a target, the redox tag is removed from the electrode, impeding the signaling current. (B) "Signal-on" E-DNA architecture based on a target-induced strand displacement mechanism. In this mechanism, target binding displaces a flexible, single-stranded element modified with a redox tag. This, in turn, strikes the electrode, generating a large increase in faradaic current. Reprinted with permission from ref 85. Copyright 2010 American Chemical Society.

on the conformational change resulting from the binding of the complementary target to the surface-immobilized DNA. As a result of this conformational change, a rigid rod-like duplex DNA is formed, moving the ferrocene label away from the electrode surface. Increasing the distance of the label from the electrode surface results in a decrease or elimination of the ferrocene signal based on the distance-dependent electron transfer property of the redox active label.

In the following years, the E-DNA sensors have been systematically improved and the progress was recently summarized in two well-written reviews.[84,85] For example, the original signal-off system (Figure 21A) was transformed into more convenient signal-on architecture (Figure 21B).[613−616] Introduction of the target-induced strand displacement method helped to achieve subpicomolar detection limits.[615] In E-DNA sensors mixed SAMs of thiolated DNA probe and mercaptohexanol (MCH) have been used. In this way, nonspecific DNA adsorption was greatly suppressed and DNA probes were helped to stand up in a position convenient for hybridization.

This mixed SAM did not, however, possess sufficient resistance to strong adsorption of proteins present in biological samples. To improve the performance of the E-DNA sensor when analyzing real DNA samples, oligo(ethylene glycol) was incorporated into the mixed SAM. This SAM showed resistance to protein adsorption without substantial interference with electron transfer across the SAM.[617] The principal requisite for E-DNA signaling is that the binding event alters the flexibility of the reporter probe and thus (a) the sensor is less susceptible to nonspecific adsorption of various components of real samples, such as blood, saliva, or urine and (b) the approach can be easily expanded to other targets. Recently, ODN or oligoribonucleotide aptamers, which changed their structure

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after target binding,[618−622] were combined with E-DNA platform, yielding a new versatile method of detection of non-nucleic acid targets.[623−644]

At present time, E-DNA sensors, which couple ligand-induced structural switching of DNAs with advanced EC detection, appear as a general platform for the development of new biosensors. There is no doubt that E-DNA hybridization sensors have some advantages as compared to approaches based only on a simple detection of duplex DNA formation (resulting from target-probe binding). On the other hand, similarly to the most of other EC DNA hybridization sensors, the rigorous comparison with current gold-standard clinical methods on authentic clinical samples is still missing.[84,85]

5.6. DNA Charge Transfer

The ability of the stacked aromatic base pairs of duplex DNA to provide a pathway for an efficient movement of charge was recognized already in 1962 by Eley and Spivey.[645] This finding did not attract much attention until 1993 when Barton et al.[646] performed experiments indicating very fast electron transfer over long distances through a so-called "π-way" of stacked bases, suggesting possible formation of a molecular wire.[647] This interesting possibility stimulated experimental and theoretical studies, resulting in the emergence of three views regarding the mechanism of long-distance charge transport in DNA:[201] (a) superexchange (charge is transported in one step by long-distance tunneling from "donor" to "acceptor" via the "bridging" DNA bases);[648] (b) multistep random walk (from donor to acceptor involving short-distance tunneling intervals linked by nucleotide sequences serving as charge "resting" sites);[649,650] (c) classical hopping (the charge resides on a one base or several adjacent bases and thermal fluctuations activate the charge motion along the DNA duplex).[650−654] Soon, tunneling appeared ineffective because of dynamic DNA structural fluctuations.[655,656] After >10 years of studies it has been shown that the dominant mechanism for charge (radical cation) migration in DNA is multistep hopping.[657−659] This mechanism involves a complex process in which the charge (resulting from one-electron oxidation of DNA) can migrate long distances by hopping through the double helix until it is irreversibly trapped in a reaction that damages DNA bases.[201] The efficiency of hopping is determined by specific nucleotide sequences. The reaction occurs usually at a guanine residue yielding primarily 8-oxoG. If no suitable guanine is available, reaction may occur at two-thymine (TT) steps in which both T's can be damaged.

In parallel, J. K. Barton and her co-workers continued their studies on DNA charge transfer based predominantly on EC methods. They developed and improved EC assays for DNA hybridization, detection of point mutations and DNA damage, as well as DNA−protein interactions, based on a charge transfer through DNA duplex.[660] Their approach mostly consisted of a preparation of self-assembled monolayers of thiolated ODN duplexes attached to the gold surface and monitoring a current from reduction of electroactive species intercalated into the duplex by voltammetry or chronocoulometry. As compared to fully matched duplexes efficiently mediating the reduction of the intercalator, the current was attenuated in presence of even a single base mismatch or lesion, demonstrating that DNA-mediated CT could be used as a sensitive tool for probing NA structure and base stacking. At first, the experiments were performed mostly with noncovalently bound intercalators (prevalently methylene blue), which were reduced either directly,[661−663] or by coupling their reduction to freely diffusing $[Fe(CN)_6]^{3-}$ in an electrocatalytic cycle,[662] but the experimental control they provided was rather limited. Nevertheless, some conclusions regarding, for example, the effect of base analogs or DNA damage products in ODN duplexes on DNA-mediated CT could be drawn.[663] CT decreased due to (a) Watson−Crick hydrogen bonding alteration, (b) addition of steric bulk, or (c) base structure modifications. On the other hand, addition of methyl group not participating in hydrogen bonding had only a little effect on resulting CT.

The DNA-mediated CT was observed also by Gooding's group,[664−666] showing a good agreement with the results of Barton et al.[660] In addition, Gooding's group showed that binding of cis-diamminedichlorplatinum(II), causing the bending of DNA duplexes, resulted in complete suppression of the EC peak due to DNA-mediated CT.[667]

Later efforts of Barton's group focused on covalently bound probes, electronically well-coupled to the base pair stack, such as cross-linked daunomycin (DM),[668−670] anthraquinone[671] (stemming from earlier works of Gooding[523,524] and Saito[672,673]) Redmond Red,[674,675] Nile Blue,[676,677] cyclometalated Ir(III) complexes,[678,679] etc. Covalent attachment of redox probes allowed controlled placement of the probe within desired sequence. Since initial experiments revealed that CT was not dependent on a distance between a gold electrode surface and a cross-linked DM,[670] different set of experiments was performed in which the length of an alkanethiol linker was varied to test its effect on the CT rate.[669] Although the CT yield was constant, irrespective of the DNA duplex length, the rate of electron transfer decreased with increased linker length, suggesting that the CT through the linker, and not through the DNA, was the rate-limiting step of the DNA-mediated reduction. Cross-linked DM was also applied in the study which showed that DNA-mediated CT occurred via the base pair stack and not through the sugar−phosphate backbone.[668] On the other hand, reduction of disulfides incorporated into the sugar−phosphate backbone of the HOPG-immobilized DNA indicated that the sugar−phosphate backbone can promote an electron transport.[680]

Until recently, efficient DNA CT through molecular assemblies over long distances has not been shown and EC measurements of DNA CT were limited to ~15 base pairs (~5 nm). Very recently, DNA CT over 34 nm has been demonstrated.[681] It has been shown that Au electrodes modified with 100-mer DNA duplex produced EC signal from a distal, covalently bound Nile Blue redox label. Presence of a single base pair mismatch in the duplex resulted in the signal attenuation similar to that observed in shorter DNA duplexes.[663,668] This result and a cleavage of the 100-mer DNA at the electrode surface by the restriction enzyme RsaI (cutting 5′-GTAC-3′) suggest that the CT is DNA-mediated and that DNA assumes native conformation at the interface, at least in cleavage-susceptible restriction site. ET rates obtained with 100-mer and 17-mer were similar, consistent thus with rate-limiting electron tunneling through the saturated alkanethiol linker.

Finding of efficient DNA CT over a distance of 34 nm brought DNA closer to the research of molecular wires and their electronic applications[682−684] and to the biological and biomedical considerations regarding the role of DNA CT in cells. In this context, it is necessary to stress that the above results obtained by J. K. Barton group represent a special case of DNA CT, which requires that the redox species (e.g., Nile Blue or Redmond Red) must be electronically well-coupled to

the DNA base pair stack. In absence of such well-coupled intercalation of the electroactive label, no DNA CT was observed. For example, nonintercalated labels (e.g., ferrocene), applied in distance-dependent DNA sensing, yielded an EC signal only when the label was located close to the electrode surface (section 5.5).[477,616] Slinker et al. admit that the full mechanism of the electrochemistry associated with the DNA-mediated ET is still elusive.[681] They postulated that DNA is conformationally gated and that the CT-active state or states are transient and at nonequilibrium.[685]

The above-mentioned studies were mostly achieved with duplexes prehybridized in solution, followed by their immobilization at the electrode surface, but the DNA-mediated CT was applied also for monitoring of in situ hybridization. For instance, a comparative in situ study at two different electrodes, to distinguish CA mismatch, was performed.[661] At one electrode, target and probe ODN were complementary, while the other electrode contained a duplex with the CA mismatch. Afterward, both duplexes were heat-denatured and the target ODNs were switched. The reversal of the EC responses from DM upon the ODN exchange indicated that the method was suitable for hybridization detection, supported by qualitatively different signals from ssDNA as a negative control. Coupling intercalator reduction to $[Fe(CN)_6]^{3-}$ electrocatalytic cycle allowed determination of 100 pM target ODN.[662] More recently, in situ duplex formation was studied by EIS during several hybridization/dehybridization steps using ferricyanide/ferrocyanide redox couple as an indicator (see also section 5.2.2).[686] Interestingly, CT occurred also via DNA/RNA hybrids, resembling the A-form, which is wider and more compact than the typical B-form.[687,688]

Charge transfer processes taking place at electrode surfaces studied by Barton's group significantly differ from those observed in solution by some other scientists.[201] In the Barton's approach, CT is related to the reduction of electroactive species intercalated into the duplex, which is immobilized at the electrode surface. In contrast, studies summarized in the Kanvah's review[201] are performed in solution and the charge resulting from one-electron oxidation of DNA can migrate long distances by hopping through the double helix until it is irreversibly trapped in a reaction that damages DNA bases. Clearly, these two approaches greatly differ. On the other hand, it is now evident that irrespective of the reductant or oxidant used to initiate the chemistry, the DNA CT can occur over long molecular distances.[33] It follows from Barton's experiments that intercalation of the electroactive compound (which may affect local DNA duplex structure)[391] is important for the observed CT. Forces acting at the electrode interface does not seem to be critical for the CT, because first observations of CT in DNA by Barton et al.[646] were done in solution without binding DNA to any surface. Problems of mechanisms of DNA CT considering the charge transfer both in solution and at electrode surfaces were recently reviewed.[33]

5.7. Detection of Single-Base Mismatches in DNA

Certain changes in DNA nucleotide sequences are in the focus of present DNA analysis because of their known relations to development of severe diseases. Among them, expansion of repetitive sequence (in neurodegenerative diseases), mutation "hot spots" (e.g., in cancer), as well as various kinds of SNPs attract special attention. At present, SNP (point mutation) in DNA can be detected as mismatched bases in DNA using various approaches. Mismatched bases are noncomplementary base-pairings, i.e., base-pairings other than the Watson−Crick pairings (Figure 4A). They can arise in vivo, for example, due to misincorporation of bases during DNA replication, 5-methylcytosine deamination, etc. Single base mismatch in the middle of the duplex ODN does not result in change of gel mobility, suggesting that mismatches may be accommodated within the helical geometry without alternation of the path of the axis in the DNA molecule.[516] On the other hand, increase in reactivity of mismatched thymine to osmium tetroxide complexes was demonstrated and utilized in their EC detection.[465,517] EC methods of base mismatch detection are scattered in different sections of this review. Here we wish only to summarize them and discuss some problems.

By hybridizing a wild type >17-mer DNA probe with mutated target DNA, a heteroduplex with a single base mismatch can be formed, which is only slightly destabilized as compared to the perfect homoduplex (without any mismatch). Straightforward EC detection of such heteroduplex based on its decreased stability at room temperature (and in weaker probe-DNA binding) is virtually impossible. Attempts have been therefore made to solve this problem (a) by finding conditions under which the difference between stabilities of the fully matched homoduplex and the mismatched heteroduplex is much larger and (b) by designing new approaches less dependent on small difference in stabilities of these two kinds of DNA duplexes.

(a) It was shown that a single-base mismatch in the DNA duplex can be identified (i) under carefully chosen temperature and ionic conditions close to the heteroduplex melting, (ii) by applying negative potential to the electrode, causing faster unwinding of the heteroduplex, (iii) using PNA as a probe. We believe that these approaches may work with model ODN targets but difficulties may arise with real DNA samples, because DNA melting temperature is strongly dependent on ionic strength (affecting electrostatic forces in the DNA double helix) and on other factors influencing hydrogen bonding and stacking in DNA. Adjusting exactly the same solution conditions in real DNA samples of the perfect and mismatched DNA duplexes may be difficult particularly in the method under (a,i).

(b) Other methods, which are less dependent on the differences in the stability of homoduplexes and heteroduplexes, can be used. They include detection of single-base mismatches by (i) DNA repair proteins, such as MutS (section 5.9.3), (ii) using chemicals, such as some Os(VIII) complexes, reacting preferentially with mismatched pyrimidine bases (section 5.2.3), (iii) detection of charge transfer through the perfect DNA duplex and attenuation of this transfer in the mismatched duplex (section 5.6), or (iv) SNP-specific incorporation of nucleotides labeled with electroactive marker (section 5.2.3).[491,517,689]

New methods have been developed[690−692] based on the assumption that nanocrystal-mononucleotide conjugates bind selectively to single base mismatches in DNA duplexes yielding characteristic voltammetric peaks. These methods appear attractive but the mechanism of the conjugate binding at the base mismatch site is unclear. Moreover, the complex resulting from such binding should be very unstable because the energy of the hydrogen bonding of NA bases in aqueous solutions is very low.[693,694] We may conclude that a number of methods for EC detection of single-base mismatches are available. It remains

to be shown which of them will be best suited for the analysis of real, biologically relevant samples.

5.8. Detection of DNA Methylation

Methylation of DNA is an epigenetic modification occurring in mammals at C5 of cytosine in CG-rich regions (called CpG islands, consisting of several tens to hundreds of CpG repetitions).[695] DNA methylation plays an important role in regulation of gene expression.[696] For instance, hypermethylation is associated with inactivation of tumor suppressor genes, leading to carcinogenesis. Methylation of CpG repeats is important not only for studies of cancer development but also for the cancer diagnostics.[697] New, fast and sensitive methods for parallel analysis of the DNA methylation are therefore sought.

In DNA methylation assays, usual techniques for detection of single-base mismatches can hardly be used since both C and mC residues exhibit the same Watson−Crick base-pairing behavior. Current DNA methylation assays are thus based on technologies capable to distinguish mC from C in DNA. Such technologies rely on various principles, including specificity of the restriction enzymes catalyzing cleavage of the DNA backbone at a specific nucleotide sequences, Southern blot,[698] methylation-specific PCR,[699] restriction enzyme-PCR,[700] bisulfite genomic DNA sequencing,[701] methylation-sensitive single nucleotide primer extension,[702] DNA microarray based on fluorescence or isotope labeling,[703] etc. Generally, methylation of C in recognition/restriction sequences may prevent the restriction enzyme from the DNA cleavage. This principle was applied in combination with restriction nuclease BStUI and PCR already in 2003 for the first EC assays of the DNA methylation.[704] The procedure, focused on the human p16Ink4a gene, was based on differences in restriction cleavage and PCR amplification of DNA from either tumor (heavily methylated) or healthy tissue. PCR-amplified DNA was detected electrochemically using $[Co(phen)_3](ClO_4)_3$ as a redox indicator. Further EC-based methods were recently introduced.[705−711]

It was mentioned above that C and mC residues exhibit the same Watson−Crick base-pairing behavior. On the other hand, the electrochemistry and chemical reactivity of C and mC need not to be the same. For example, on treatment with bisulfite, the C residue is readily deaminated and transformed to U while mC resists such treatment. This difference was utilized in the bisulfite genomic DNA sequencing[701] and has been also applied in EC detection of the DNA methylation in combination with other techniques, such as PCR.[699] Moreover, both C and mC are reducible at Hg electrodes, while U is nonreducible (in aqueous solutions). Several decades ago, it was shown that the DPP reduction peak of DNA decreases as a result of the bisulfite treatment.[712] To our knowledge, this finding has not been utilized in the present EC analysis of the methylation of DNA.

Treatment of DNA with bisulfite followed by PCR was used to develop EC assay for the DNA methylation in the promoter region of cyclin D-dependent protein kinase inhibitor, p16 gene (p16Ink4a).[711] After this treatment, PCR converted unmethylated C to T and mC to C. 20-mer DNA probes were designed for methylated (C) and unmethylated (T) base residues and immobilized on EC array composed of 25 Au electrodes. Ferrocenylnaphthalene diimide (FND, threading intercalator binding tightly to dsDNA) was used as a redox indicator of the DNA hybridization. Twenty ng of methylated sample obtained from the methylation-specific PCR was sufficient for the determination of the methylated DNA in presence of 10-fold excess of unmethylated DNA, as documented by EC signals from the two DNA probes. This assay was recently improved by using newly synthesized redox indicator, the naphthalene diimide derivative (F4ND), carrying four ferrocene moieties.[707] It was shown that F4ND bound to dsDNA in the threading intercalation mode despite its bulky substituents. Application of F4ND resulted in improved discrimination between fully matched and mismatched DNA duplexes and allowed experiments at lower concentrations of the DNA samples as compared to the previous work with a less complex FND.[711]

The above methods were based on the established ways of determination of methylated DNA, which were adapted for EC analysis. It can be expected that other approaches will be developed, based on the specific EC and chemical properties of methylated DNA. Recently, it has been shown that the DNA intrinsic oxidation signals of C and mC appear at different potentials, with E_p of mC being about 150 mV less positive than that of C (Figure 22).[709] These results were obtained using nanocarbon film created by electron cyclotron resonance sputtering method producing the film structure which differs from that of heterogeneous BDD or amorphous diamond-like carbon.[713] This nanocarbon film electrode has interesting properties including an atomically flat surface, a wide potential window, and little surface fouling, being thus attractive for EC analysis of various biomolecules.[714,715] As compared to glassy carbon and BDD, nanocarbon film provided mC signals which were well-distinguished from C and the potentials of A, G, T, C, and mC did not overlap (Figure 22). The proposed method of mC determination was simple and rapid, however micromolar ODNs were needed for good resolution of the oxidation peaks.

In another label-free determination, methylated DNA was adsorbed at the HMDE surface and a SWV peak CA (due to reduction of C and A, section 3.2) was measured.[708] dsODN duplexes (21- to 24-mers) with short ss overhangs containing 6 C and 4 mC residues in their upper strands (lower strands were not methylated) produced SWV cathodic peaks only slightly smaller than those obtained with nonmethylated (10 C) ODNs. On the other hand, CpG methylated human male Jurkat cells (from acute T-cell leukemia) showed a significant decrease of the peak with only 10% of mC in the molecule. DNA containing 100% methylated C residues displayed only negligible peak. These results appear very interesting, unfortunately their interpretation is not straightforward because of some drawbacks, such as (a) the composition of the background electrolyte (50 mM phosphate, 0.3 M NaCl, pH 6.9) was not convenient for measuring reduction currents of C and A at the given pH (section 3.1);[114,118] (b) lack of background curve to compare it to the 100% modified DNA curve; (c) lack of data on MW of Jurkat DNA samples with different extents of methylation and lack of data on conditions for reaching the surface coverage with individual Jurkat DNA samples, etc. In spite of these drawbacks, the new approach appears interesting, deserving further attention.

5.9. Double-Stranded Target DNAs

Specific recognition of DNA sequences is usually based on interaction of two complementary ssDNA strands forming a duplex (Figure 2). Almost all papers dealing with the EC detection of the DNA hybridization are based on this principle. In experiments with natural tDNAs, their denaturation is

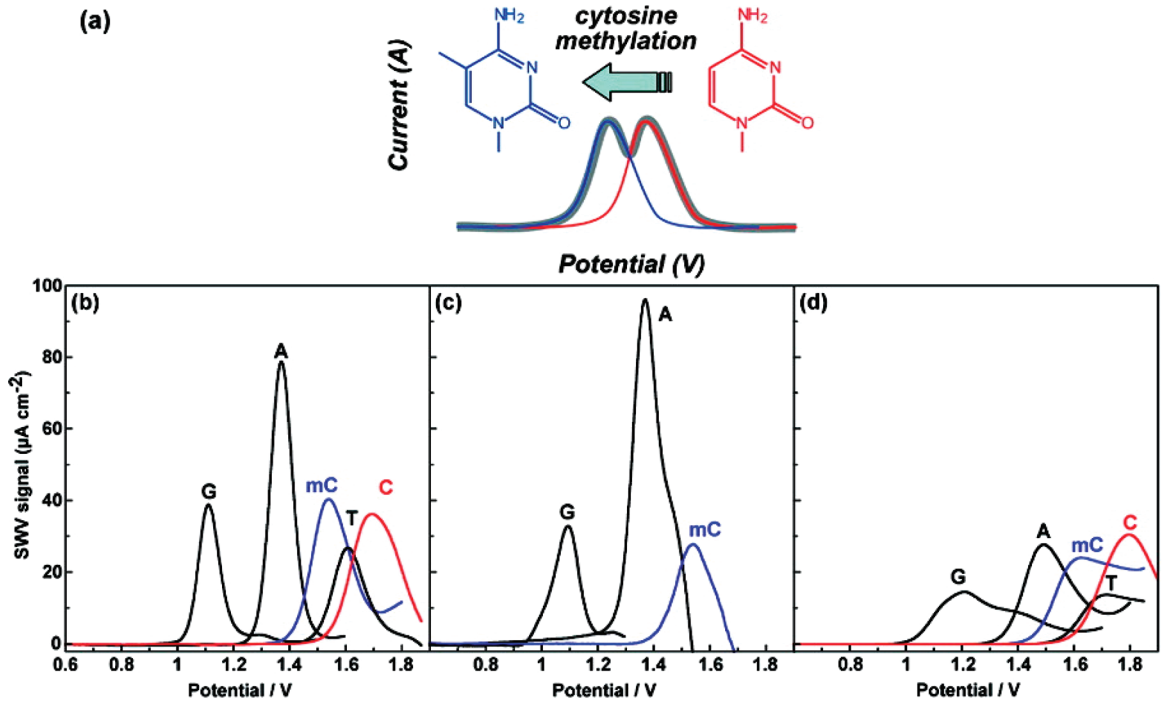

Figure 22. EC detection of cytosine methylation. (a) Structure and EC response of methylcytosine, as compared to cytosine. (b-d) Background-subtracted SWVs of 100 μM nucleosides at (b) nanocarbon film formed by electron cyclotron resonance sputtering method, (c) glassy carbon, and (d) BDD electrodes, respectively. Methylcytosine could be distinguished also within short oligonucleotides (n = 6). Reprinted with permission from ref 709. Copyright 2008 American Chemical Society.

necessary to generate the tDNA single strands, which are then hybridized with the probe ssDNA. In some cases the denaturation step can be avoided and duplex tDNA can be directly analyzed (a) when formation of a DNA triplex is detected,[716,717] instead of the usual duplex detection, (b) if low MW sequence specific minor groove binders[718] or (c) sequence- or DNA structure-specific proteins are used.[719−721]

5.9.1. Triplex Structures. Formation of the triple helix in biosynthetic polynucleotides was first reported in 1957 by Felsenfeld, Davies and Rich.[722,723] In recent decades, formation of inter- and intramolecular triplexes was intensively studied.[308,724,725] EC detection of various structures of biosynthetic polynucleotides by means of Hg electrodes was popular in the 1960s and 1970s.[113,114,298] Later, triplex formation based on G oxidation peak at carbon electrodes and anodic peak of G and cathodic peak of C residues at HMDE was studied.[716] Using these signals, it was possible to detect polynucleotide triplex formation in solution. On the other hand, polynucleotide interactions at the HMDE surface resulted in low yield of duplex or triplex structures. In contrast, at the surface of carbon electrodes sequence specific interactions took place, but instead of triplex formation observed in solution, a mixture of structures, including duplex and triplex, were detected.

Recently, Patterson et al. reported E-DNA sensor (section 5.5) for ds tDNA based on redox-tagged triplex-forming ODNs (TFO) as recognition element for dsDNA.[717] Upon the addition of the relevant dsDNA target, the probe formed a triplex (via reverse Hoogsteen base pairing in the major groove), which inhibited electron transfer between the probe's redox moiety and the gold electrode producing a decrease of the oxidation peak (Figure 23). Signaling of the presence of the target was thus similar to that used in the signal-off E-DNA sensors working with ss tDNAs. After demonstrating the principle of this approach with 22-mer polypurine TFO sequence and a synthetic ds tDNA, a 19-base polypyrimidine TFO probe was used to interact with complementary polypurine tract in dsDNA (conserved in all HIV-1 strains). In this way, ds tDNA was detected at concentrations as low as ~10 nM. In addition, unpurified, ds PCR amplicons containing the relevant HIV-1 sequence were successfully detected. This new approach in the EC DNA sequence sensing is limited to polypurine/polypyrimidine sequences but it appears very interesting and worth of further development. Application of PNA strongly interacting with dsDNA could be particularly interesting.

5.9.2. Sequence-Specific DNA−Protein Binding and Detection of Point Mutations. DNA-binding proteins play important roles in many biological processes, such as replication, transcription and DNA repair.[14,726] They bind to DNA in different modes, including structure- and sequence-specific as well as nonspecific (predominantly electrostatic) binding. Different methods have been used in studies of DNA−protein interactions, particularly the high-resolution X-ray crystal analysis, NMR and a number of various biophysical methods. On the other hand, methods of EC analysis were applied in these studies to a lesser degree,[483,719,720,727−732] if we do not include numerous aptamer-related papers summarized in many reviews[733−743] which are covered in this paper only in relation with some of the described EC methods. Considering the protein and DNA electroactivity, as well as recently elaborated methods of protein and NA electroactive labeling, relatively small number of EC studies of the DNA−protein interactions is surprising. Here we wish to briefly discuss a question of how the DNA−protein interactions can be utilized in EC sensing of specific nucleotide sequences and base mismatches using dsDNA as a target.

5.9.3. Detection of Point Mutation (Single-Base Mismatch) in dsDNA by Means of MutS Protein. MutS

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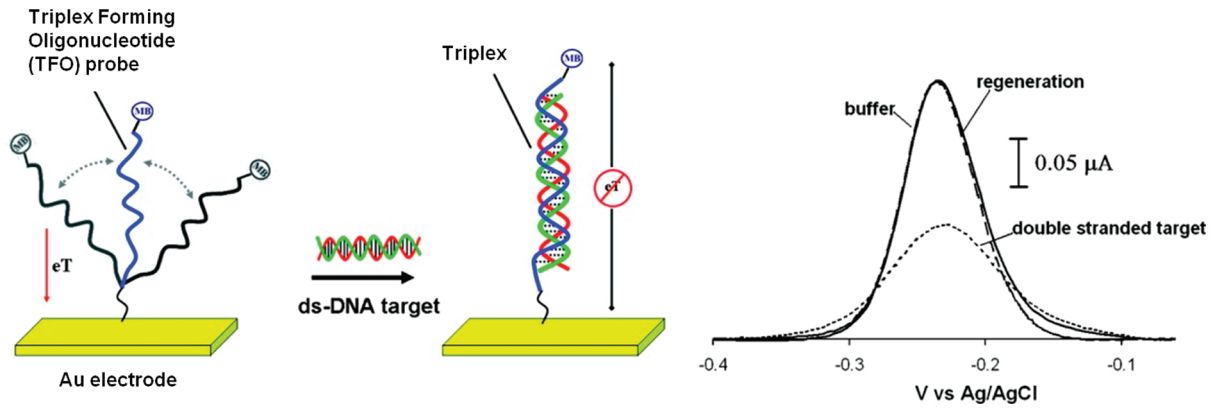

Figure 23. E-DNA sensor employing a triplex-forming oligonucleotide (TFO) probe for detection of double-stranded DNA targets. (Left) The sensor consisting of a polypurine or polypyrimidine TFO probe modified at its 3′-terminus with a methylene blue redox tag and at its 5′-terminus with a mercaptohexanol moiety for attachment on a gold electrode. (Right) The faradaic current arising from the flexible TFO probe is significantly reduced in the presence of the double-stranded DNA target. Triplex formation presumably reduces the efficiency with which the terminal redox tag collides with the electrode surface and transfers electrons. Reprinted with permission from ref 717. Copyright 2010 American Chemical Society.

protein plays a role in the DNA repair systems and recognizes unpaired and mispaired bases in duplex DNA. Recently, this protein has been utilized in detection of point mutations in vitro using radioactively or fluorescence-labeled MutS protein,[744] protection of DNA from exonucleolytic cleavage,[745] electrophoresis,[746,747] quartz crystal microbalance,[748] or EC methods.[720,731,732,749−752] For instance, a label-free EC analysis made use of a biotinylated dsDNA, which was attached to the surface of magnetic microbeads and interacted with MutS protein in solution.[720] After dissociation of the protein from the DNA-MutS complex, the protein was adsorbed at HMDE and determined using the electrocatalytic CPS peak H. This peak forms due to the catalytic hydrogen evolution reaction involving amino acid residues with labilized protons (i.e., lysine, arginine, histidine, and cysteine close to neutral pH),[253,753] enabling determination of very low protein concentrations.[753−755] In this way, single base mismatches and insertion/deletions were recognized by MutS in dsDNA and the protein was detected by CPS down to tens of attomole (pg) amounts. The sensitivity of the MutS determination at carbon electrodes was by about 3 orders of magnitude lower.[752] In theory, this principle might be used to direct detection of point mutations arising in cells. A lot of work would be, however, necessary to materialize this principle in practice.

Recently, new methods have been proposed based on protein[731,750] or DNA[749] immobilization on gold electrodes. Either formation of the DNA-MutS complex[731,749] or dsDNA alone were detected electrochemically.[750] Cho et al. immobilized His-tagged MutS protein at a gold electrode coated with (1S)-N-[5-[(4-Mercaptobutanoyl)amino]-1-carboxypentyl] iminodiacetic acid (HS-NTA) and employed cyclic voltammetry, EC quartz crystal microbalance and EIS to determine the binding affinity of mismatched DNA duplexes to the immobilized MutS protein.[731] Different binding affinities of MutS protein to mismatched DNA were confirmed (GT > CT > CC >AT) and it was shown that even CC mismatch, which is weakly bound to MutS, could be detected. Chen et al. studied interaction of mismatched DNA with MutS protein immobilized at a gold electrode using gold NPs. They detected mismatched DNA bound by MutS protein using methylene blue as a redox indicator.[750] Detection limit as low as 0.6 pM of mismatched DNA was reported. Gong et al. offered a label-free method for the detection of single nucleotide mismatches.[749] In their system, thiolated DNA was immobilized at a gold electrode and formation of the complex of mismatched DNA with MutS was detected by EIS. In the presence of a single base mismatch at the top of the dsDNA layer, MutS was bound to the DNA, producing changes in the charge transfer resistance observed in Nyquist plots (section 5.2.2).

5.9.4. Sequence-Specific Binding of p53 Protein to dsDNA. Tumor suppressor protein p53 binds sequence-specifically to the DNA consensus sequence (CON) located usually within promoters of genes whose expression it modulates.[756,757] To detect the CON, reverse mode of the above strategy[720] for detection of the DNA mutations was used.[719] By another words, p53 protein was immobilized at the magnetic microbeads (using p53-specific monoclonal antibodies) to interact with DNA in solution and the DNA complexed with the p53 protein was determined electrochemically. Linear DNAs were dissociated from the complex using increased salt concentration and determined using ACV at HMDE. Competition experiments were used to determine supercoiled DNA, which resisted high salt treatment. To increase sensitivity of the determination of DNA, tail labeling using terminal deoxynucleotidyl transfererase and modified deoxynucleoside triphosphates was developed.[483] 3-nitrophenyl-7-deazaG was selected as the most useful label. It was shown that p53 protein recognized and bound to the CON within the labeled DNA. Reduction of the nitro group yielded a well-developed voltammetric peak close to −0.5 V.

In principle, two approaches have been used in EC analysis of proteins in which either (a) immobilized DNA interacted with the protein in solution followed by EC detection (i) of the dissociated protein (section 5.9.3) or (ii) of the DNA conformational change induced by the DNA−protein interaction (section 5.5) or (b) immobilized protein interacted with the DNA in solution followed by EC detection of the dissociated DNA, which was either labeled[483] or unlabeled.[719] Sensitivity of the label-free electrocatalytic protein detection should be much better than that of label-free DNA detection, based on oxidation or reduction of the DNA bases. Label-based methods have not been yet fully exploited and their sensitivity will depend on the nature of the label.

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5.10. Concluding Remarks

Some aspects of the recent development of the DNA hybridization sensors were briefly summarized above. They include applications of (a) inosine-substituted capture probes for measurement of intrinsic DNA oxidation signals, (b) enzymes or nanoparticles in sandwich assays, (c) carbon nanotubes as unique immobilization support, (d) magnetic beads for hybridization and separation or (e) molecular beacons responding to target binding, etc. Although many of them brought considerable improvements in terms of sensitivity or specificity, more work need to be done to overcome challenges that we are facing, including interference of species in complex matrices, stability of the sensors or their reusability. Nevertheless, great progress made in recent years provided important insights into mechanisms and resulted in development of some biosensor devices (section 8). In the following section, we shall deal with problems of EC analysis of real, biologically relevant NA samples.

6. ANALYSIS OF BIOLOGICALLY-RELEVANT SAMPLES

Among thousands of papers aimed to development of the DNA hybridization sensors (Figure 1), a great majority dealt with ODN targets, that is, with the targets whose analysis is highly improbable in practice. Such experiments were justified in the early stage of the EC sensor development and can be still important if some new principles or approaches are shown (e.g., new ways of NA labeling, transducer engineering, signal amplification, etc.). Using a target ODN containing nucleotide sequence of some gene important in medicine and claiming detection of the given gene is at this stage of little importance and can be misleading.

Recognition of two cDNA strands is a delicate event in the DNA hybridization. DNA renaturation of whole viral and (usually fragmented) bacterial chromosomal DNAs in solution can be finished at properly elevated temperature and convenient ionic strength within hours.[3] On the other hand, nonrepetitive sequences of chromosomal eukaryotic (such as calf thymus or human) DNA will not renature even after several days. Determination of specific single copy sequences in human DNAs without PCR amplification is thus rather difficult. For example, meeting of 20-mer DNA probe with its complementary sequence among 3×10^9 base pairs of the human genome cannot proceed very fast.[7] Also, detection of the probe-target duplex in abundance of $>10^8$ base pairs of noncDNA is a difficult task. Once the DNA probe meets its complementary sequence in the ss tDNA and form a short duplex DNA segment, the problem will arise how to detect this duplex in very large excess of noncomplementary ssDNA. To our knowledge, no well-done EC analysis (containing all necessary control experiments) of a single-copy sequence in DNA of mammals without PCR amplification has been reported. Thus any way which can speed up the hybridization step or increase the specificity and sensitivity of the EC detection should be followed and exploited (see also Table 3 for detection of real NA samples based on ECPs).

6.1. PCR-Amplified DNAs

At this stage of development of the EC hybridization sensors, amplification of tDNA by PCR represents an indispensable step in the DNA sequence analysis of genomic DNA and particularly of the human DNA.[758,759] In recent years, there has been a tendency to develop point-of-care (POC) molecular diagnostics devices bringing the DNA testing to the vicinity of the patient.[45,69] POC are particularly important for resource-limited regions lacking readily accessible centralized laboratories. While centralized laboratories can still rely on fluorescence detection of DNA, EC-based methods have a great potential to develop into simple and portable POC devices including both PCR amplification and EC detection of DNA.

6.1.1. End-Point Detection of PCR Amplicons. Established post-PCR detection methods, such as blotting and gel electrophoresis of DNA, are time- and labor-consuming, inconvenient for POC analysis. In contrast, EC DNA analysis offers simple operation, high sensitivity, easy miniaturization, and low cost, being thus a good candidate for application in POC. A pair of ODN primers used in PCR ensures specific amplification of DNA. These primers can be labeled and the labels can be then utilized in the EC DNA detection. Biotin-labeled primers have been frequently used because small biotin molecules do not interfere with the DNA amplification and offer highly sensitive EC detection of amplicons, using avidin/streptavidin complexes with enzymes or specific antibodies.

For the detection of PCR-amplicons, high sensitivity of detection methods can help to decrease the number of PCR cycles (and save time and expenses), but in this case sensitivity is not critical because the amount of amplified DNA is usually sufficient even for moderately sensitive EC analysis. Also the requirements for the electrode surface screening against nonspecific adsorption are less strict because only the DNA polymerase and rests of primers and nucleoside triphosphosphates (not incorporated in the amplified DNA) may represent the potential interferences in the EC analysis of the PCR amplicons. In contrast, EC analysis of DNA in various biological matrices (such as blood, saliva, or urine) without PCR amplification is substantially more difficult with much higher requirements for both sensitivity and specificity of the EC analysis.

To our knowledge, end-point EC detection of PCR amplicons started about 10 years ago[408,422,429,760,761] and quickly developed in recent years using label-free[424,427,558,762−766] and label-based methods,[458,711,767−774] as well as DST platforms.[529,775,776] Label-free methods were based mainly on electroxidation of G residues at carbon electrodes,[427,762−764] on the detection of purine bases released from DNA by acid treatment followed by determination at carbon[213] or Hg electrodes,[408] or on AC impedance measurements.[558,766] Various redox indicators noncovalently bound to dsDNA by intercalation,[711,770,771] groove binding[768,769,772,774] and electrostatic modes[458,773] were used for EC amplicon detection. Covalently bound electroactive labels[422,466,777] and nanoparticles,[769,778] as well as enzymes based on biotin-streptavidin/avidin binding[529,617,776,779,780] and immunoassays[781,782] yielded high sensitivity of the amplicon determination. Other ways, such as E-sensors (molecular beacons, see section 5.5) or ECP-based assays (Table 3), were utilized in the detection of PCR-amplified DNA. In addition to the EC amplicon detection based on surface-immobilized DNA probes, several immobilization-free schemes were recently developed.[474,774] For example, ferrocene-labeled PNA (Fc-PNA) hybridized in solution with tDNA.[474] The negatively charged Fc-PNA/DNA hybrid was attracted to the positively charged electrode producing a higher EC signal than free Fc-PNA, while opposite results were obtained at negatively charged electrode.

It is also possible to amplify cDNA copies of RNA. In a method called reverse transcription PCR (RT-PCR), RNA template is first hybridized to an ODN primer, followed by an

extension using an RNA-dependent DNA polymerase to create cDNA copy, which can be further amplified by PCR.[11] Until recently, only few EC detection-based assays employing RT-PCR were reported.[529,783,784]

6.1.2. Trinucleotide Repeat Expansion. Expansion of trinucleotide repetitive sequences (triplets) is associated with various genetic neurodegenerative diseases, for example, Huntington disease (triplet CAG·CTG), Friedreich ataxia (GAA·TTC), fragile X syndrome (CGG·CCG), myotonic dystrophy (CTG·CAG), etc. Detection of triplet expansion, currently mostly performed with Southern blotting or PCR-assisted gel electrophoresis, is critical for their early diagnosis. Some papers appeared also on EC detection of triplets involving PCR amplification.[430,785] For instance, triplets coding both for myotonic dystrophy and for fragile X syndrome were PCR-amplified and immobilized on the ITO electrode, where the oxidation of G using an electrocatalysis by $[Ru(bipy)_3]^{2+}$ was monitored.[430] The catalytic currents due to the oxidation of immobilized G residues increased with the number of repeats and were a linear function of the repeat number after normalization to the number of immobilized strands. Quantification of triplets involved, however, radiolabeling of the fragments, and the method required a presence of G in the triplet.

In another work, the triplet expansion specific for the Friedreich ataxia was detected electrochemically (without radioactive labeling), using DST and two different labels - Os,bipy and alkaline phosphatase.[785] The strategy was based on detecting the PCR-amplified tDNA which contained not only the GAA triplet, but also naturally occurring A stretch. The A stretch was used to hybridize with oligo$(dT)_{25}$ covalently attached to PMBs. Pyrimidine residues in the tDNA were modified with an electroactive Os,bipy, and the signal of the DNA-Os,bipy adduct was independent of the $(GAA)_n$ length, enabling monitoring of the number of captured tDNA molecules. The $(GAA)_n$ length was characterized by employing biotin-labeled oligo(CTT), complementary to the GAA triplet, followed by an addition of streptavidin-alkaline phosphatase complex, enzymatically converting electroinactive substrate to an EC-detectable signal.

6.1.3. Real-Time PCR Amplicons Detection. The literature summarized above shows that EC end-point detection methods are now well established. On the other hand, application of EC detection in real-time PCR (section 6.1.3), which enable quantitative measurements of the DNA copy numbers during the amplification cycles, has started only in recent years.

Unlike end-point detection (section 6.1.1), real-time PCR aims to quantitatively measure initial copy numbers of DNA analyte and provides immediate information on the kinetics of the PCR. Currently, the real time PCR uses commercially available thermocyclers coupled with fluorescence detection, which amplify and measure the DNA concentration simultaneously.[11] On the other hand, several EC assays were also developed.[186,522,786−789] The first electrochemistry-based real-time PCR technique involved a solid-phase extension of the surface-immobilized capture probe, into which a Fc-labeled dUTP was incorporated during the PCR.[522] With increasing number of PCR cycles, more Fc-dUTP was accumulated at the electrode surface, leading to a signal enhancement. This technique was later implemented to a microchip.[786] Another EC technique for monitoring real-time PCR amplicons was based on the use of intercalators.[787,788] With the increasing amount of double-stranded PCR products generated in each PCR cycle, fewer and fewer intercalator molecules (free in solution) diffused to the electrode and underwent redox processes, thus leading to a current decrease. This method did not involve immobilization of the probe; postlabeling of the duplexes was, however, necessary, and the "signal-off" architecture limited a number of cycles during which the method could be used. There is also an option to electrochemically detect oxidation of 7-deazaguanine, which can be enzymatically incorporated by PCR (see section 3.1.4 for more details).[186,789] Recent progress in this field was reviewed.[45]

6.2. Combination of Biochemical and Electrochemical Approaches

Are we limited only to the detection of PCR-amplified NAs? Although attempts to improve EC sensing of PCR-amplified NAs are at present still very important, significant progress has been made in the EC analysis of NAs without the PCR amplification, especially due to sophisticated combination of biochemical and electrochemical approaches.

6.2.1. Genomic DNA. As mentioned above, detection of single-copy gene sequences in eukaryotic genomes is a difficult task. On the other hand, some genes can occur in the genome at high copy number, resulting from gene duplication events and EC detecting of their sequences may be thus less difficult. Designing an ODN capture probe complementary to an intergenic region composed of repeated elements, such as satellite DNA, tandem repeats or mini- and microsatellites, consisting of blocks of nucleotides repeated in the genome, might help to take advantage of natural DNA amplification. Thus capturing a multicopy intergenic sequence located next to a single-copy gene might help to detect this gene. Highly sensitive EC techniques and thorough biochemical considerations are however necessary, including application of bioinformatics, choice of proper restriction endonucleases and eventually other enzymes and biochemicals, etc. Some papers claiming detection of specific sequences in genomic DNA without PCR amplification[772,790−794] were recently reviewed.[53]

6.2.2. Sequences Inserted Into Plasmids. Plasmids are extra-chromosomal, usually circular dsDNA molecules (much shorter than chromosomal DNAs), occurring naturally in bacteria (see also section 4.2.4). Recombinant plasmids, constructed for molecular biological research, occur in bacterial cells in a high copy number and many of them are commercially available. Nucleotide sequence inserted into the plasmid is naturally amplified in bacteria and can be analyzed by EC methods. Purification of plasmid DNAs can be facilitated by commercial kits. cdDNA molecules are cleavable by enzymes, including restriction nucleases. After such cleavage these DNAs can be thermally or alkali denatured to form ssDNA suitable for hybridization. Construction of a label-free EC sensor for detection of 401 bp human interleukine-2 (hIL-2) DNA inserted into the plasmid pET21a(+) expression vector was recently reported.[764] Capture probes were immobilized on pencil graphite electrodes charged to positive potential. The 20-mer ODN probes contained only one G residue to make easier the detection of G oxidation signal of tDNA. 5438 bp long plasmid pET21a(+) without the hIL-2 insert was used as a control. Electrochemistry was also used to detect DNA damage in plasmid DNA and to monitor ligation of previously cleaved cdDNA (section 4.2.5). Simple inexpensive EC device for fast checking of nucleotide sequences inserted in plasmid can be useful in molecular biology laboratories involved in frequent cloning experiments.

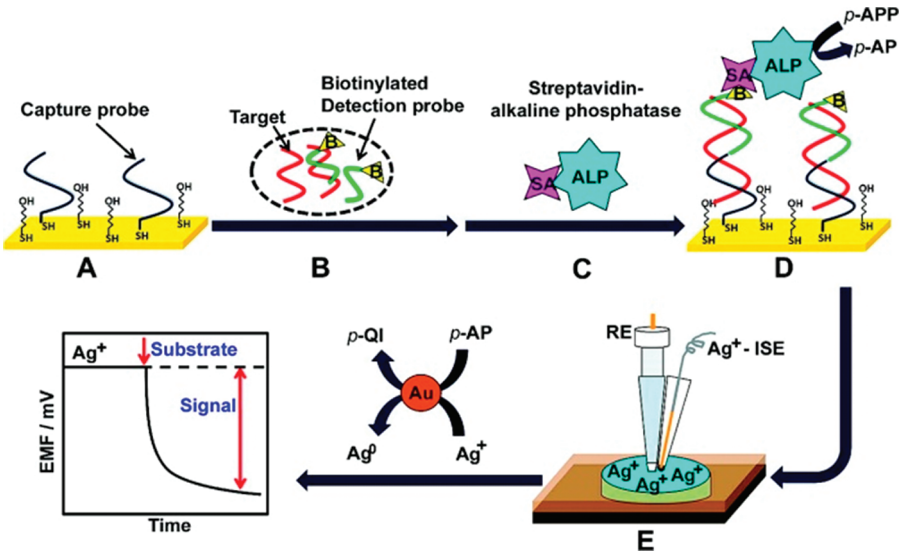

Figure 24. Representation of the potentiometric detection of DNA hybridization with ion-selective electrode (ISE). (A) Formation of the mixed thiol monolayer (thiolated DNA capture probe and mercaptohexanol, MCH) on the gold substrate; (B) hybridization of the target DNA/biotinylated reporter probe mixture with the surface capture probe; (C) binding of the streptavidin-alkaline phosphatase (SA-ALP) complex; (D) addition of the ALP substrate, *para*-aminophenylphosphate (*p*-APP), to initiate the enzymatic reaction, and (E) potentiometric detection of changes in the level of the silver ion upon adding an aliquot of the enzymatic reaction mixture to the Ag^+-ISE cell. Later, potentiometric detection was replaced by amperometry.[497,530] Reprinted with permission from ref 496. Copyright 2009 American Chemical Society.

6.3. Ribonucleic Acids

Ribosomal RNA (rRNA) genes are present in high copy numbers in the genome and are suitable for EC detection. In recent years, a significant progress has been achieved in EC detection of bacterial rRNA sequences.[414,461,496,497,530,780,795−803] For example, *E. coli* contains between 5×10^3 and 2×10^4 copies of 16S rRNA per cell.[804] Such a large number of RNA copies are detectable by EC DNA sensors working with femtomolar detection limits (see below) and the analysis can be performed using raw bacterial lysates from actual body fluids such as urine, representing thus an important advance compared to previous studies. In this paragraph, we wish to summarize recent progress in EC detection of uropathogen rRNA, discuss new possibilities in EC studies of some messenger RNAs (mRNAs) and pay special attention to the detection of microRNAs (miRNAs).

6.3.1. Analysis of Uropathogen rRNA Sequences. In 2001, a group from UCLA pioneered EC detection of bacterial rRNA without PCR amplification.[805] They took advantage of multiple copy rRNA and the microelectromechanical system (MEMS) technology; this technology made it possible to integrate micrometer-sized mechanical parts with electronics and production process employing lithography.[806,807] In the bacterial lysate, biotinylated ODN probes (ODNb) were used for solution-phase hybridization with bacterial rRNA. Fluorescein-conjugated reporter probe (ODNf), complementary to a different sequence of the bacterial rRNA, was used to form an ODNb−rRNA−ODNf sandwich. This sandwich was bound to the streptavidin-coated gold working electrodes of the MEMS detector array, followed by addition of peroxidase-conjugated antifluorescein antibody and hydrogen peroxide as the enzyme substrate. The sensitivity of the determination was increased by linking the HRP/H_2O_2 system with a redox soluble TMB, enabling catalytic cycling. In this way, determination down to ∼1000 of bacterial cells was possible.[805]

In the following years, this approach was modified and greatly improved in Haake's[461,795−798] and Wang's[414,496,497,530] laboratories. For instance, very low detection limit of 10 CFU (Colony-Forming Units) in a potentiometric assay (Figure 24) for detection of 16S rRNA of *E. coli* pathogenic bacteria in the 4 μL sample was obtained,[496] but its further improvement was not possible without increasing the S/N. Such improvement (see below) was recently achieved by sophisticated chemical engineering using new ternary SAMs for better interfacing the electrode surface, preventing nonspecific adsorption and decreasing the noise.[497] Also, potentiometric detection has been recently replaced by amperometric detection.[497,530]

6.3.2. Improved Shielding of Electrode Surfaces by Binary and Ternary SAMs. During the years of the development of EC DNA sensors, several schemes for attaching NA probes onto electrode surfaces have been suggested. Among them, thiol binding has been frequently utilized to immobilize ODN capture probes on the Au electrode surface.[187,808]

6.3.2.1. Binary SAMs. Target recognition relied on the binary mixed SAM using thiolated ssODN and a diluent. Mixed SAMs of thiol-derivatized probe with MCH have been frequently favored.[375,439,809] This SAM minimized nonspecific adsorption via the polar −OH head groups of MCH and ensured "stand up" position of the immobilized probes (Figure 25A). In addition to MCH,[343,395,808,810−812] other diluents were applied: 2-mercaptoethanol,[813] 4-mercaptobutan-1-ol,[439] 2-mercaptoundecanoic acid,[814] or 11-mercaptoundecanol.[813] It was shown that the length of the alcohol-terminated diluent thiol has a large impact on the time taken to form a perfect duplex on the electrode surface.[666]

Mixed monolayers of thiolated DNA with MCH were originally designed to displace any weakly adsorbed DNA bases off the gold surface.[187,808] The ability of binary SAMs to sufficiently protect the electrode from the nonspecific adsorption of interfering components of biological samples was questioned by some authors[439,809,815] due to incomplete backfilling and consequent lower reproducibility in the analysis of biological samples. Moreover, the binary MCH SAMs appeared not sufficiently resistant to protein adsorption.[816]

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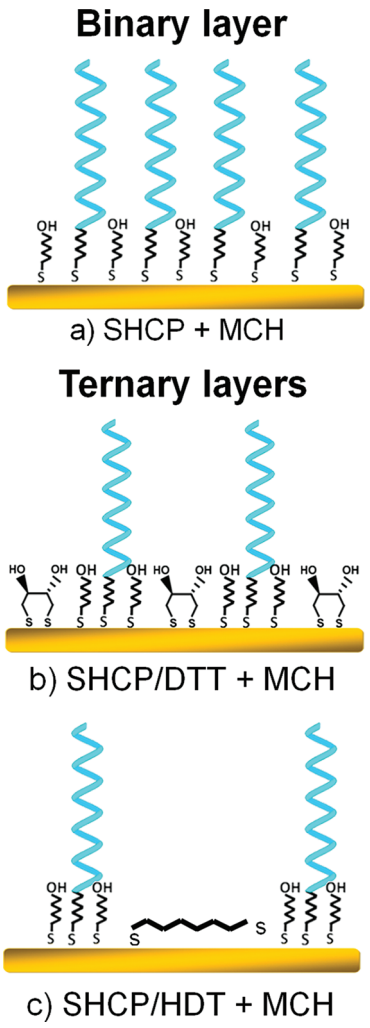

Figure 25. Schematic illustration of the (A) conventional binary layer composed of a thiolated capture probe (SHCP) and mercaptohexanol (MCH); (B) new ternary SHCP/DTT + MCH and (C) SHCP/HDT + MCH monolayers. DTT, dithiothreitol; HDT, hexanedithiol. Adapted with permission from ref 530. Copyright 2011 Elsevier.

Incorporation of thiol-terminated oligo(ethylene glycol) into ODN probe SAM resulted in some improvement in shielding of the electrode against the nonspecific adsorption.[617,816−818] Another binary SAM composed of bipedal polyethylene glycolated ssODN with various diluents was recently proposed and characterized.[819] Further improvement resulted from introduction of ternary SAMs.[497,817,818]

6.3.2.2. Ternary SAMs. Recently, a multicomponent ternary mixed SAM was introduced using SH-ssODN, 3-mercaptopropionic acid (MPA) and MCH, constructed by sequential adsorption on the 100 nm thick gold-coated Si wafer.[818] This surface was characterized using AC impedance, chronocoulometry, X-ray photoelectron spectroscopy and surface FTIR techniques, indicating anisotropic "head-to-head" group hydrogen bonding in MPA−MCH forms and optimum ODN probe density. Using this ternary SAM, 10 pM detection limit was achieved. Substantially better detection limit was achieved with a new ternary SAM, consisting of coassembled thiolated capture probe (SHCP), MCH and dithiothreitol (DTT) showing remarkable resistance to nonspecific adsorption (Figure 25B).[497] Using this highly compact SAM, detection limit of 40 zmol of rRNA (in 4 μL samples) and 1 CFU *E. coli* per sensor was reached. Interestingly, DTT, the new member of the ternary SAM, produced pinhole-free SAM at HMDE, allowing detection of the protein-catalyzed hydrogen evolution at the DTT-modified HMDE by means of constant current chronopotentiometry.[253,254] Further improvement of the SHCP/MCH+DTT ternary layer was achieved when MCH was coimmobilized with hexanedithiol (HDT) instead of the DTT, leading to 8-fold improvement in S/N characteristics over DTT (Figure 25C).[530] S/N for 1 nM DNA target obtained with HDT was ~350, one of the highest so far achieved. The new SHCP/MCH+HDT layer was sufficiently resistant to nonspecific adsorption even in undiluted serum or urine, allowing quantification of the target DNA down to tens of attomoles in these complex media. To our knowledge, this is the first report of using undiluted serum or urine during the DNA hybridization. Moreover, the chip with such ternary layer displayed favorable antifouling properties, as demonstrated by measurements performed after 24 h and one week.

6.3.2.3. Double-Surface Technique. Further papers were published, dealing with multicopy bacterial rRNAs.[414,801,802] Among them, LaGier et al.[414] applied the DST (used earlier in DNA analysis; see section 5.1) based on sensitive stripping analysis of purine bases obtained after NA acid hydrolysis. They used probe-modified magnetic beads to incubate them with either RNA extracts or lysates of *E. coli* cells (from 109 CFU). Captured RNA was hydrolyzed and G was determined at graphite electrode using DPV stripping; nonspecific adsorption to the beads was negligible.

6.3.3. mRNA. The copy number of a specific mRNA in a particular cell type varies drastically from one mRNA to another, and depends on a series of factors usually related to the function of the protein for which that mRNA codifies. In general, the copy number of a particular mRNA in a cell extract is much lower than the copy number of rRNA molecules. Detection of specific mRNAs without prior amplification is thus a challenging task.

mRNAs can serve as cancer biomarkers in the body fluids, important for early cancer detection. Already in 2004, an EC method was proposed for detection of expression of breast cancer susceptibility genes (indicated by mRNA), such as p53, HSP90, BRCA1, and histone H4 without a reverse transcriptase-PCR amplification.[820] mRNAs were extracted from both healthy and cancer human breast tissues and total mRNA was labeled with cisplatin-coupled biotin conjugates. Labeled mRNAs were hybridized, followed by incubation with avidin−glucose oxidase. The electrode surface (in a microarray) was overcoated using a cationic redox polymer containing osmium−bipy complexes. The oxidation current of glucose was detected amperometrically. The method was validated by comparing its results with conventional method for gene expression quantification (ribonuclease protection assay), showing good agreement for the tested genes. The lowest amount of mRNA detectable by this method not requiring PCR amplification was about 1.5 ng. Shortly afterward, another method was reported[451] for detection of the mRNA of the p53 tumor suppressor gene in rat liver tissues. Detection of a point mutation in mRNA (forming a single base mismatch with the probe DNA) was possible.

Detecting cancer biomarkers directly in saliva is less unpleasant to patients than in blood. Recently, a new method of EC detection of low-copy number of salivary IL-8 mRNA without PCR amplification was proposed.[371] This method was based on principles of EC molecular beacons (section 5.5) and

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coupled an enzymatic amplification (using antifluorescein-HRP conjugate) with target-induced DNA conformational change in the Au surface-immobilized hairpin probe. Steric hindrance prevented binding of HRP to the fluorescein-labeled end of the hairpin probe located close to the electrode surface (Figure 26).

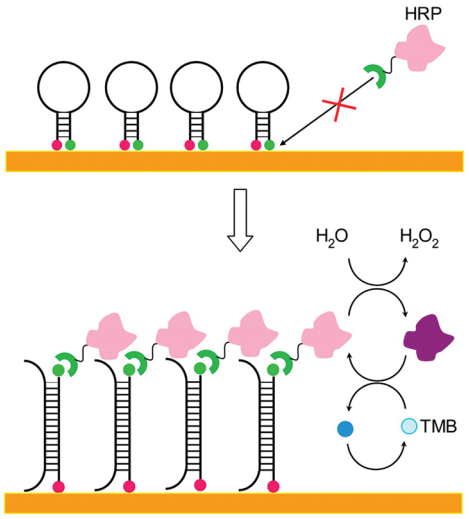

Figure 26. EC detection of mRNA by specific signal amplification in hairpin probe. The biotin label (red circle) bound to streptavidin served as an anchor to the chip surface, and the fluorescein label (green circle) allowed for binding of the antifluorescein-HRP complex. When no target is bound to the probe, the hairpin is closed, thus the complex does not form and no signal is observed (top). After hybridization with the target, the hairpin opens up and the HRP complex is formed (bottom). TMB regenerates the reactive HRP system, thus amplifying the current signal. Reprinted with permission from ref 371. Copyright 2008 Oxford University Press.

Only as a result of binding of the complementary RNA target, the hairpin was opened, assuming a duplex structure in which the fluorescein-labeled end of the probe was far away from the surface, accessible to the HRP binding. HRP-generated EC signal was detected amperometrically with a detection limit down to 0.4 fM.

6.3.4. MicroRNAs. MicroRNAs (miRNAs, also called RNA interference, RNAi) are a class of small endogenous 21−25 nucleotide-long noncoding RNAs.[821−824] In the time of its discovery by Lee et al.[88,821,825,826] in 1993, miRNA was initially thought to be a sporadic anomaly of nature. After several years, miRNAs have emerged as key post-transcriptional regulators of gene expression. The coding sequences for miRNAs are typically found in intergenic regions (spaces between genes in which there are few to no sequences coding for proteins) or within the introns of protein-coding genes. By base pairing to mRNAs, miRNAs can mediate translational repression, playing an important regulatory role in animals and plants. miRNAs have been shown to participate in the regulation of almost every cellular process investigated so far. miRNA-based regulation is implicated in disease etiology and has been tested for treatment. There is a growing evidence of links between miRNA expression and the onset of cancer and other diseases. Some miRNAs can function as tumor suppressors, while others can act as oncogenes, either directly or indirectly. Recently, several preclinical and clinical trials have been initiated for miRNA-based therapeutics.[87,821,826,827] Currently, miRNAs are predominantly analyzed using RT-PCR, Northern blot and microarray analysis with optical detection. Only recently, new methods have emerged which are not associated with standard RT-PCR and Northern blotting. Novel methods based on various ways of detection, including colorimetric, fluorescence, EC, and bioluminiscence reduce procedural complexity and expenses.[87,88]

EC methods, either label-free[434] or involving miRNA labeling,[828−831] have been proposed. Recently published label-free method[434] is based on G oxidation signal of C-free miRNAs after hybridization with G-free probe at carbon electrodes. The use of G-free probe limits thus application of this method to the detection of C-free miRNAs. The authors claim that their method could be a potential tool for routine detection of miRNA-122 in serum and biopsies of hepatological patients, but they show hybridization only with synthetic ODNs. More work will be necessary to show that this method can be used in real miRNA analysis.

Labeling of miRNA offers better selectivity and sensitivity but application of ferrocene labels (frequently used in DNA detection) has some limitations, because they cannot be applied for labeling of miRNAs directly in a biological matrix. They can be, however, useful in sandwich assays, in which prelabeled reporter probes can be used. A pioneer miRNA assay employing electrocatalytic nanoparticle tags was reported by Gao and Yang already in 2006.[830] RNA extracts were treated with periodate, reacting with the 3′-end ribose of RNA yielding 3′-end dialdehydes. After hybridization, isoniazid-capped OsO_2 nanoparticles were brought to the electrode through a condensation reaction with the 3′-end of periodate-treated miRNAs. Such miRNA-modified electrode exhibited electrocatalytic activity toward the oxidation of hydrazine, drastically reducing its oxidation overpotential. In this way specific miRNAs were detected in total RNA extracts of HeLa cells.

In further papers, Gao and Yu[828,829] used two different transition metal complexes as tags, instead of OsO_2 nanoparticles, for RNA labeling. The transition metal complexes used were either covalently ligated to the 3′ end of miRNAs[828] or coordinated to their purine bases.[829] The tags showed catalytic activity toward the oxidation of ascorbic acid[828] or hydrazine,[829] shifting the oxidation potential negatively by as much as 600 and 850 mV, respectively, and greatly enhancing the oxidation peak current. Total RNA was extracted from HeLa cells, and the RNA extracts were enriched in miRNAs and other short RNA species using a centrifugal filter device with a cellulose membrane.

Fan et al. utilized conducting polymer nanowires (see also section 5.4) for EC miRNA quantification.[606] They immobilized electrically neutral PNA capture probes onto a nanogapped array made up of 100 pairs of interlocking comb-like microelectrodes. A total RNA sample from HeLa and lung cancer cells was hybridized, creating a net negative charge at the capture probe. After incubation with a mixture of aniline/HRP/H_2O_2 at pH 4.0, the protonated aniline molecules aligned around the negatively charged, hybridized miRNA, forming polyaniline nanowires. These nanowires were then expanded by doping with HCl vapors, creating an electrically conducting network bridging the gaps of the biosensor array (Figure 27). Since the total deposition of these nanowires and their electrically conducting network was dependent on the amount of hybridized miRNA, a calibration curve was constructed, correlating the recorded conductance to the concentration of miRNA. Dynamic range from 20 pM to 10 fM and a detection limit of 5 fM were reported. The major improvement of this

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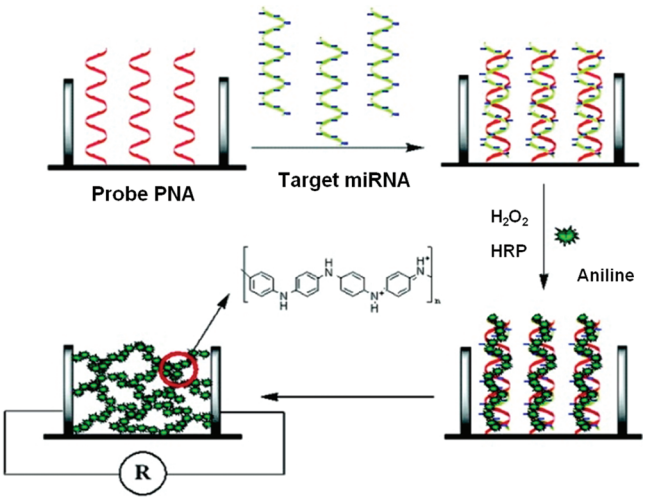

Figure 27. Schematic illustration of a biosensor for miRNA detection. The total RNA sample was hybridized to the capture probes, creating a net negative charge. The hybridized miRNA was then incubated with an aniline mixture. The protonated aniline molecules aligned around the hybridized miRNA forming polyaniline polymers. The nanowires were then expanded by doping with HCl vapors, creating an electrically conducting network bridging the gaps of the biosensor array. Reprinted with permission from ref 606. Copyright 2007 American Chemical Society.

assay was due to the PNA capture probes, which have a much higher affinity for miRNA due to the absence of electrostatic repulsion, as demonstrated earlier in DNA–PNA interaction studies.[281,392,458,832] This method yielded results in terms of sensitivity and selectivity not previously observed with miRNA EC detection and it offered advantages over optical detection methods, including less expensive electronics, easy sample preparation and smaller sample quantities.

Another multiplex EC chip, based on a pattern of gold on the surface of a silicon wafer and electrodeposited palladium providing highly nanostructured microelectrodes (NMEs), was designed.[833] The NMEs modified with PNA probes were exposed to total RNA for hybridization and the duplex formation was assayed with a redox reporter system,[458,540,834,835] which relied on the accumulation of Ru(III) on the NA immobilized at the electrode surface. The signals were amplified by the inclusion of ferricyanide and regeneration of Ru(III) chemically after its EC reduction.

Recently, a gap hybridization assay based on four components was described for EC detection of miRNAs.[831] As a result of binding of complementary miRNA to a gap between capture and reporter probe, the reporter esterase enzyme (EST-2) was brought to the vicinity of the electrode and produced enzyme-mediated EC signal. The gap hybridization assay demonstrated selective detection of miRNA-16 within a mixture of other miRNAs, including discrimination of single base mismatch. Detection limit of 2 pM (or 2 amol) of miRNA-16 was reported. miRNA-16 and miRNA-21 were detected in parallel, and higher expression of oncogenic miRNA-21, as compared to miRNA-16, was demonstrated in human breast adenocarcinoma cells. Using EST2 as a reporter enzyme was convenient because of easy site-specific binding to ODNs and the enzyme thermostability, allowing hybridizations at elevated temperatures.

The above label-based methods are rather complex, involving many steps and/or complex electrode systems. Recently, a simple method of end-labeling of RNAs with electroactive marker was proposed.[836] Ribose at the 3′-end of 22-mer oligonucleotides was selectively modified by complex of six-valent osmium and 2,2′-bipyridine [Os(VI)bipy], producing two CV redox couples at pyrolytic graphite electrode. Using SWV, these oligonucleotides were detected down to 250 nM. At mercury electrodes the Os(VI)bipy-oligonucleotides adducts produced electrocatalytic peak at ~−1.2 V, allowing their determination down to picomolar concentrations. High specificity of Os(VI)-bipy for ribose in NAs and high sensitivity of the determination at mercury and solid amalgam electrodes give promise for new simple methods of miRNA determination. This method differs from earlier DNA and RNA modification with osmium tetroxide complexes with nitrogenous ligands. Such complexes were applied for end-labeling of DNA and PNA using carbon,[462–464] mercury,[250,464,470] and gold[466–469] electrodes, as well as for electroactive labeling of proteins[837] and peptides[838] (section 5.2.3). Labeling of miRNA with these Os complexes is possible but modification of bases would interfere with the miRNA hybridization. Os(VIII)bipy-modified DNAs were highly immunogenic making possible easy generation of poly- and monoclonal antibodies.[283,839] Antibodies against Os(VI)L-RNA adducts have been recently generated to be applied in the analysis of miRNA and other types of RNA.

7. DETECTION OF DNA DAMAGE

Highly sensitive methods of DNA damage detection, based on differences of surface denaturation in covalently closed and open circular DNA molecules at mercury electrodes, were summarized in section 4.2.5. Already by the end of the 1950s, it was shown that DNA damage could be detected electrochemically.[840,841] DNA was isolated from X-ray irradiated and control rats and increase in the guanine oscillopolarographic signal was observed. Using DPP, changes in peak II (section 3.2, Figure 4) were observed in DNAs enzymatically digested or X-ray irradiated in vitro.[133,842]

In the following paragraphs, EC DNA damage detection with solid electrodes not containing Hg will be summarized. More information can be found in reviews.[313,843–845]

7.1. Strand Breaks in DNA

Compared to Hg electrodes[314] (section 4.2.5), the sensitivity of strand break detection with solid electrodes is lower, unable to provide information about the formation of one ssb per plasmid DNA molecule. Labuda and co-workers have developed a redox indicator-based method for detection of DNA double helix disruption using intercalators binding specifically to immobilized dsDNA and producing a redox signal at carbon electrodes. Upon DNA degradation, the indicator signal decreased due to its lower affinity toward ssDNA.[846–848] The method was applied for studying DNA damage induced by nitrofluorenes,[849] tin(II) or arsenic(III) compounds,[850] or hydroxyl radicals,[851] or for evaluation of antioxidative properties of various flavonoids.[852]

7.2. Damage to Bases in DNA

Solid electrodes were successfully applied to monitor oxidative damage of bases or deeper DNA degradation, including fragmentation of longer DNA molecules, release of monomeric components, etc. For instance, oxidative damage is caused mostly by ionizing radiation or by an attack of ROS. Currently, the resulting oxidation products are determined by optical or EC methods coupled with previous LC or CE separation techniques, which make the determination laborious and rather

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expensive. Thus more time- and cost-saving methods are sought, one of which could be EC biosensing assays without any separation techniques involved.

Guanine, which is the target for a wide spectrum of genotoxic agents, is also the most easily oxidized base, producing well-measurable signals at mercury and carbon electrodes. Using voltammetry or chronopotentiometry, guanine oxidation peak at carbon-based electrodes, G_{OX}, was monitored upon exposure of the DNA to a variety of genotoxic agents, including antitumor platinum complexes[853] hydrazine derivatives,[854] aromatic amines and phenol compounds,[855,856] aflatoxins,[857] arsenic oxide,[858] chromium,[199] or peroxynitrite.[200] Moreover, irradiation by UV[859,860] or γ-rays[198] was also studied.

Most of these label-free strategies involved measurement of peak G_{OX} decrease after the damage, but this "signal-off" approach is inherently limited in sensitivity. Also, experimental error for carbon-based electrodes can reach up to 10%, and thus a minor damage (which causes only small change in G_{OX} peak) may not be detectable. Relatively large portion of G residues (about one G among 10−20 intact G's) must be damaged for reliable EC response.[317]

Improved sensitivity is achieved when the damage leads to a formation of a new product, giving rise to previously unseen signal. Such a product is, for example, 8-oxoguanine, resulting from DNA oxidative damage which can be electrochemically detected at carbon, ITO, or platinum electrodes.[861−864] For instance, Brett's group has utilized the signal specific for 8-oxoguanine for detection of DNA damage by antineoplastic drug adriamycin,[865] flavonoid quercetin[866] or chromium,[199] and Thorp's group has employed redox catalytic mediator, $[Os(bipy)_3]^{2+}$, selectively oxidizing 8-oxoguanine but not guanine, enabling determination of 8-oxoguanine-containing DNA.[862] Detection limits lower than 1 nM of 8-oxoguanine were obtained with amperometry in combination with HPLC even in a presence of strong interferent, uric acid. Measurements were also performed in urine samples, with detection limits of around 80 nM.[867] Damage to DNA bases can be enzymatically transformed to ssb's, and detected at Hg-containing electrodes at high sensitivity (detection of one damaged base among $\sim 10^5$ intact ones, section 4.2.5, Figure 11).

Rusling's group developed an interesting approach for monitoring reactive metabolites by employing electrodes coated with cytochrome P450/DNA films prepared using a layer-by-layer technique.[868−872] In these sensors, enzyme reaction produced metabolites and the resulting DNA damage was detected by either voltammetry using soluble $[Ru(bipy)_3]^{2+}$ catalyst (regenerated by A and G residues in damaged DNA),[868,871] or with single electrode ECL[869,870] and ECL arrays.[872,873] The required films could be placed on a single PGE,[869,874,875] in a block array format,[872] or on silica nanoparticles for LC-MS analysis.[876] Studies performed on these formats included the metabolism and genotoxicity of styrene,[868] benzo[a]pyrene,[873] *N*-nitrosamines,[872,876] and the genotoxicity of arylamines activated by N-acetyltransferase.[877]

8. BIODEVICES: DNA MICROARRAYS AND LAB-ON-A-CHIP

Undoubtedly, current efforts in biosensing research focus on developing a device capable of highly sensitive, parallel and reliable detection of DNA or RNA in fast, simple and automated assays. For that purpose, various biochips (also called DNA chips or microarrays, containing many reaction sites for parallel analysis) have been utilized in analysis of expression of thousands of genes.[50,878−884] An ultimate goal might represent a construction of lab-on-a-chip (LOC, or Micro Total Analysis System, μ-TAS), a biodevice integrating DNA sensor technology into microfluidic system, believed to perform an automated and complete assay, including sample preparation, PCR amplification, or EC detection.[68,90,94,885] It is thus not surprising that besides a predominant optical detection, there have been numerous attempts to couple DNA chips with an EC detector, providing benefits in terms of lower cost, faster assays, smaller device dimensions, or compatibility with microfabrication technology.[94] Biodevices (both optical and electrochemical) still face several technical barriers, including problems with sensitivity, stability, reproducibility, or limited shelf life.[93]

Nevertheless, several commercially available EC biodevices were introduced in recent decade, giving a huge promise for future endeavors. For instance, GenMark Diagnostics (U.S.A.) offers eSensor Technology, integrating microfluidics and EC detection with the assay time of only 30 min.[886] The signal indicating hybridization event is generated voltammetrically using ferrocene-labeled reporter probe. CombiMatrix Diagnostics (U.S.A.) has developed oligonucleotide microarray platform containing 12 544 individually addressable microelectrodes in a semiconductor matrix.[887,888] This approach requires labeling of the target DNA with biotin, which (after hybridization with microarray capture probe) binds HRP-streptavidin conjugate. The HRP-catalyzed enzymatic reaction involves an oxidation of TMB, followed by amperometric determination of the product, reaching subnanomolar detection limits. An interesting strategy for analysis of DNA base composition is by passing a single DNA strand through a protein nanopore.[889] Oxford Nanopore Technologies (U.K.) chemically engineered one of the membrane proteins, α-hemolysin, to enhance the sensor effectivity capable of distinguishing all major DNA bases.

Some recent attempts to create functional DNA chips or LOC devices (not yet commercialized) are summarized in Table 4, along with one of the schemes showing a strategy for parallel detection of DNA samples (Figure 28).

9. SUMMARY AND CONCLUSION

After >50 years of its existence, electrochemistry of NAs is a booming field, currently aimed at developing DNA sensors and sensing assays. A huge amount of knowledge on NA interactions with electrically charged surfaces summarized in this review makes electrochemistry of NAs potentially useful in various fields of biochemical research. DNA and RNA, as well as their mimetics, such as PNA, are electroactive species, producing oxidation and reduction signals of their bases at some electrodes (sections 3.1 and 3.2). Moreover, these NAs can produce capacitive signals related to their adsorption/desorption behavior (section 3.3). Both the faradaic and capacitive signals reflect changes in the DNA structure under conditions close to physiological; highest sensitivity to small structural changes was observed with mercury and solid amalgam electrodes. Using proper EC methods and ionic conditions, either (a) the secondary changes in the DNA structure at the electrode surface can be eliminated to obtain information about the DNA structure in solution, or (b) structural changes can be induced by prolonged contact of dsDNA with electrically charged surface, followed by their EC detection. Application of negative charges to the surface-attached DNA may result in the DNA denaturation and eventual strand

Table 4. List of Several Articles Published between 2007 and 2010 Focused on DNA Chips/Microarrays and LOC Devices

type of chip	EC method	target NA	LOD[a]	ref
16 AuE chip	CA	*Escherichia coli* 16S rRNA	8 fM	497
8 AuE chip	CA	human breast adenocarcinoma miRNA	2 pM	831
4 AuE chip	DPV	herpes simplex virus, Epstein−Barr virus, cytomegalovirus	5 aM	890
silicon chip with 16 AuEs	CV	*Alu1* restriction enzyme site	N/A	677
16 Pt-microdisk electrode chip	CV	microbial rRNA targets of different lengths	<100 pM	582
interdigitated microelectrode array on PMMA surface	CM	*Cryptosporidium parvum*	single oocyst	891
48 AuE array	SECM	*Salmonella* spp.	100 fM	892
flow-through EC-qPCR microfluidic device	SWV	489 bp gene fragment	N/A	787
CMOS-based 4 × 4 AuE	CV	human retinoblastoma 1 mRNA	50 pM	893
integrated microfluidic Ti/Au sensor	ACV	*Salmonella enterica*	10 aM	894
interdigitated AuE array in silicon chip	CA	*Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, ortho pox viruses	N/A	895
ImmuChip	CA	hazelnut genomic DNA	200 pM	776
ITO-coated Si glass microchip	DPV	bacteriophage M13	$\sim 10^3$ copies/μL	786
AuE-containing LOC	ACV	human H1N1, avian H5N1	400 nM	896
interdigitated Pt microelectrodes-based LOC	EIS	*Salmonella choleraesuis*	10 nM	897
conducting polymer/AuNP on Pt microelectrode array	LSV	100-bp DNA ladder	113 fM	898
CE-based microfluidic device with Au microelectrodes	HDV	8-hydroxy-deoxyguanosine	100 nM	899

[a]ACV, AC voltammetry; AuE, gold electrode; CA, chronoamperometry; CE, capillary electrophoresis; CM, coulometry; CMOS, complementary metal−oxide−semiconductor; CV, cyclic voltammetry; DPV, differential pulse voltammetry; EC, electrochemical; HDV, hydrodynamic voltammetry; ITO - indium tin oxide, LOD - limit of detection, LSV - linear sweep voltammetry, PMMA - poly(methyl methacrylate), SECM - scanning electrochemical microscopy.

separation at the electrode surface (section 4.2). At positively charged surfaces, no denaturation was observed and stabilization of DNA at these surfaces was reported. However, it is unclear whether DNA assumes a double-helical structure at surfaces or whether the DNA duplex adopts a ladder-like or some other, more or less unwound structure prior to its opening at negative potentials (section 4.4).

Label-free methods (based e.g., on the intrinsic electroactivity of NAs) are simple and convenient, but in many cases DNA labeling offers better sensitivity and other advantages. Covalently bound electroactive labels can be easily introduced in NAs (section 5.2). Some labels (such as ferrocene) can be bound to ODNs during their (usually commercial) synthesis in the organic chemistry laboratories. Os(VIII) complexes can be introduced into DNA, RNA, and PNA by addition to the 5,6-double bond in pyrimidine bases, performed just by mixing the reagent with NA at room temperature. Different labels can be also attached to DNA during its enzymatic synthesis. DNA labeling is particularly important for specific end-labeling of target or reporter probe DNAs. In the recent decade, the NA labeling was greatly influenced by application of nanotechnologies (section 5.3).

First papers on NA electrochemistry were published >50 years ago, but for about 30 years DNA electrochemistry was a small field involving handful of laboratories, publishing in average ~10 papers per year. Starting from 1990, an exponential increase in a number of papers occurred, mounting to >700 papers per year during the recent years (Figure 1). This large increase is related to the progress in genomics and particularly in the Human Genome Project, requiring new methods for parallel DNA nucleotide sequencing. EC methods arrived to this field later than optical methods, but their outlook for practical application appear bright, because their performance is now comparable to optical methods; yet EC methods are simpler, less expensive, easily adaptable for miniaturization and well-suited for decentralized analysis and inclusion into LOC.

During the first 30 years, the electrochemistry of NAs dealt mainly with basic EC problems, such as electroactivity and adsorption/desorption of NAs, but also with DNA structure in solution and at interfaces (producing early data on DNA premelting and polymorphy of the DNA double helical structure[138] in agreement with trends in the DNA research in that time). In spite of this orientation, many early steps important in the present development of the EC DNA sensors were done. For example, application of solid carbon electrodes,[139] covalent labeling of DNA,[126−128] invention of DNA-modified electrodes,[125] detection of DNA renaturation[100,102,104] and DNA damage[133,840,841] etc., were published before 1990 (Table 2).

The development of EC biosensors for DNA hybridization (nucleotide sequencing) started with rather primitive methods using carbon and gold electrodes in combination with redox indicators (binding preferentially to dsDNA). Alternatively, label-free detection based on guanine oxidation signals at carbon electrodes, or later G oxidation with a mediator at ITO electrodes, was used. At gold electrodes, DNA was attached to the surface via its terminal −SH group, forming a SAM with standing-up DNA molecules. At carbon electrodes, unlabeled probe DNA was lying flatly at the electrode surface. These techniques worked relatively well with synthetic ODN targets. They were, however, mostly poorly efficient in the analysis of real DNA samples. To improve the abilities of EC analysis of DNA in biological matrices, about 10 years ago the DST was proposed, in which the DNA hybridization was performed at one surface (usually magnetic beads, optimized for capturing target DNA or RNA from biological materials) and EC detection of the DNA hybridization was done at another surface, that is, at the detection electrode best suited for the given electrode process (section 5.1). DST offered very high sensitivity and specificity in the analysis of real DNA samples, but it required more manipulation than usual SST or an efficient microfluidic system.

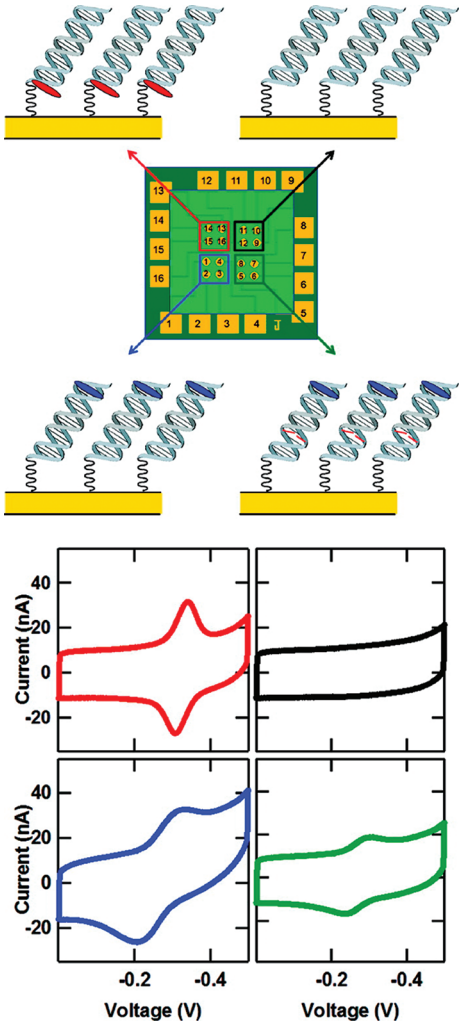

Figure 28. Multiplexed detection on the DNA-modified chip. (Top) Illustration of a chip layout with four distinct DNA target complementary strands. (Bottom) Cyclic voltammetry data from each of the four different DNA targets depicted in the top figure. The four sequences consisted of a well-matched strand with a well-matched strand with a proximal 3′ Redmond Red probe (red), a well-matched strand with a distal 5′ Nile Blue redox probe (blue), a well-matched strand with no redox probe (black), and a 5′ Nile Blue labeled strand containing a single base-pair (CA) mismatch (green). Reprinted with permission from ref 677. Copyright 2010 American Chemical Society.

In the second half of the 1990s, Barton et al. demonstrated unique charge transfer between methylene blue intercalated in the assembly of 15−20 base pair duplex DNAs and a gold electrode to which the duplex was attached via thiol tether (section 5.6). In the following years, this system was improved and employed in the design of various EC assays including DNA hybridization and single-base mismatches. Recently, similar charge transfer has been shown with 100-mer DNA duplex containing covalently bound Nile Blue redox label. Presence of a single base mismatch attenuated the EC signal similarly as in earlier studies of shorter DNA duplexes.

In 2003, a new type of a DNA sensor (called E-DNA sensor) was proposed in Heeger's laboratory, based on a change in the structure of ferrocene-labeled DNA hairpin probe into a linear duplex, resulting from the DNA hybridization, resembling thus the molecular beacons based on optical detection (section 5.5). In the hairpin probe, the ferrocene label was located close to the electrode surface and produced an EC signal. Upon the interaction with complementary target DNA, the hairpin changed into a duplex and the label was moved away from the surface, diminishing the EC signal. Later, this signal-off technique was improved and transformed into a more versatile, signal-on technology.

Most of the above techniques are strongly dependent on the nature of the electrode used for the DNA sensing. In the ECPs, this dependence is less strict and the performance of the ECP sensor is more dependent on the nature and way of ECP polymerization. ECP may play a passive role, serving just for DNA immobilization, but it can also directly influence the transduction process, manifested by a change of the ECP conductivity, redox behavior, etc. (section 5.4). In such cases, using electrically neutral PNA as a probe is very convenient, because PNA binding to negatively charged target DNA results in a large, easily detectable change in the electrical properties of the DNA·PNA duplex.

Papers on development of the DNA sensors dealing with synthetic ODN targets displayed reasonable performance. Real EC analysis does not, however, work with such ODNs and analysis of genomic DNA sequences mostly requires amplification of tDNA by PCR. Compared to synthetic ODN targets, PCR amplicons are usually longer and may contain some additional substances, such as nucleotides and proteins. A number of EC methods have been developed, suitable for the analysis of PCR-amplified DNA and RNA (section 6.1). Moreover, attempts have been made to use EC analysis in end-point detection of PCR amplicons (section 6.1.1) and to replace optical detection in real-time PCR by EC detection (section 6.1.3). Analysis of NAs without PCR amplification is much more difficult than the detection of PCR amplicons and represents a challenge. In recent years, significant progress has been done, including analysis of unamplified uropathogen rRNA, as well as messenger and microRNAs (section 6.3), frequently based on combination of EC and biochemical approaches. Analysis of unamplified real NA samples is rather difficult because it has to be done in complex biological matrices, such as cell culture, blood, saliva, or urine and requires very high sensitivities and signal-to-noise ratios. S/N has been recently greatly increased by improved shielding of gold electrode surfaces by binary and ternary SAMs. Also DST has shown good properties in the NA analysis in complex biological matrices. Combination of efficient shielding of the surface (at which tDNA is captured) with sandwich assay using TMB as a substrate for HRP/H_2O_2 oxidation appear now very useful in analysis of PCR-unamplified, biologically relevant NA samples (section 6).

Cytosine methylation plays important roles in various diseases, including cancer. Simple DNA hybridization techniques cannot be used to detect methylated cytosine, because both base residues exhibit the same base pairing behavior. Attempts have been made to adapt specific methods of mC analysis for EC detection or to develop EC methods based on differences in EC properties of C and mC in DNA (section 5.8). More work will be however necessary to further develop this promising field.

Interaction of the ssDNA probe with complementary ss tDNA followed by detection of the duplex DNA is a critical step in most EC DNA hybridization sensors and assays. It has been shown that in some cases this scheme can be replaced by direct analysis of natural dsDNA (without its denaturation/ssDNA formation). This can be done (a) with DNA sequences forming easily triplex structures (e.g., homopurine·homopyrimidine sequences), (b) using sequence- or dsDNA structure-specific proteins, or (c) using low MW compounds, such as

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sequence-specific minor groove binders. The approaches under (a) and (b) have been recently utilized in the EC DNA sensing. The possibility of direct analysis of natural ds tDNAs appears interesting, and it can be expected that it will attract attention of scientists involved in DNA EC analysis.

This review shows a great progress in electrochemistry of NAs and in development of EC DNA sensors and DNA sensing assays. Determination of any nucleotide sequence, including those containing point mutations (section 5.7) or determination of the length of repetitive sequences (e.g., in neurodegenerative diseases, section 6.1.2) is not a problem. Mostly, PCR amplification is, however, necessary, but in some cases naturally amplified RNA and DNA sequences can be determined without PCR. Determination of single copy mammalian gene without PCR represents still a challenge. Another challenge can be seen in development of new EC methods for studies of NA-protein interactions based on the NA sequence- or structure-specific protein binding. About 6−7% of eukaryotic genome encodes DNA-binding proteins.[726] Such proteins play a central role in many biological processes, such as replication, transcription, DNA repair and packaging, etc. Combination of electrochemistry with this area of biochemistry and molecular biology may yield both new EC tools for biochemistry and biomedicine and knowledge about properties of DNA−protein complexes in solution and at interfaces. Such EC tools need not rely only on signals of DNA but also on protein signals (both protein intrinsic and label-based signals) (section 5.9). In recent years, a significant progress has been done in development of EC methods of aptamer (functional NAs) analysis, including those for NA-protein binding. Such aptamers are mentioned in this review only if they are based on principles of the discussed DNA hybridization sensors (e.g., sections 5.5 and 5.9).

The experiments, which in the first decades of the DNA electrochemistry contributed to the DNA research, were done with liquid mercury electrodes and showed extreme sensitivity for changes in DNA structure. Later, solid electrodes were preferred, such as gold and carbon ones, which were better suited for DNA sensors than liquid mercury. There is no doubt that solid electrodes greatly contributed to the present development of the DNA hybridization sensors. But should we forget about Hg electrodes in electrochemistry of NAs? These electrodes differ from most of the solid ones by their range of working potentials. For example, in 0.2 M sodium acetate, pH 4.8, Au electrode offers its potential window between −0.91 and +1.49 V while HMDE works within −1.70 and +0.31 V.[245] Moreover, a number of SAEs are available, including meniscus, polished and ultrathin sputtered electrodes.[245] Potential windows of SAEs are similar but not identical to HMDE. For example, polished silver SAE works between −1.51 and +0.31 V, while meniscus gold SAE between −1.47 and +0.31 V.[245] Thus substantially more negative potentials can be reached with Hg-containing electrodes than with gold and carbon electrodes. Reaching such negative potentials is important in measuring EC signals due the catalytic hydrogen evolution reaction (CHER). It has been shown that CHER signals of NAs, carbohydrates,[900,901] as well as of peptides[902] or proteins[753,755,903] (either labeled or unlabeled) can be measured not only with HMDE, but also with silver SAE,[255] but not with electrodes not containing Hg. Solid amalgams are frequently used in the tooth filling and their toxicity can be neglected. Moreover, the amount of mercury in a small tooth filling corresponds roughly to at least 10^5 of ultrathin sputtered SAEs.[245,255] We may thus conclude that SAEs should be considered as a new type of solid electrodes for biosensors, including the NA sensors. HMDEs, which offer better reproducibility than any solid electrodes, are in their present form of little use in DNA sensors. Unique properties of liquid mercury electrodes, including atomically smooth, hydrophobic and highly reproducible surface make them, however, irreplaceable tools in the EC research of biomacromolecules. High sensitivity of these electrodes for small changes in NA structures (section 3.2.3) has been recently extended to changes in protein structures, both at HMDE and SAEs.[254,904] It can be thus expected that within soon, Hg-containing electrodes will be more frequently used in laboratories dealing with EC analysis of biomacromolecules.

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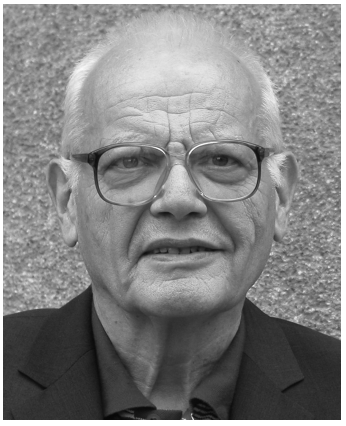

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LIST OF ABBREVIATIONS

ACV	alternating current voltammetry
AdS	adsorptive stripping
AdTS	adsorptive transfer stripping
AFM	atomic force microscopy
BDD	boron-doped diamond
bipy	bipyridine
cdDNA	circular duplex DNA
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony-forming units
CHER	catalytic hydrogen evolution reaction
CNT	carbon nanotube
CPS	chronopotentiometric stripping
CSV	cathodic stripping voltammetry
CT	charge transport
CV	cyclic voltammetry
DE	detection electrode
DET	direct electron transfer
DM	daunomycin
DME	dropping mercury electrode
DNA	DNA
dNTP	deoxynucleotide triphosphate
DPP	differential pulse polarography
DPV	differential pulse voltammetry
dsDNA	double-stranded DNA
DST	double-surface technique
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EC	electrochemical
ECL	electrochemiluminescence
ECP	electronically conducting polymer
EIS	electrochemical impedance spectroscopy
EVLS	elimination voltammetry with linear scan
Fc	ferrocene
FITC	fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
GCE	glassy carbon electrode
HDT	hexanedithiol
HMDE	hanging mercury drop electrode
HOPG	highly oriented pyrolytic graphite
HRP	horseradish peroxidase
ITO	indium tin oxide
LOC	lab-on-a-chip
LSV	linear sweep voltammetry
MB	methylene blue
MCH	mercaptohexanol
MEMS	microelectromechanical system
MF-SAE	mercury-film modified SAE
miRNA	microRNA
MPA	mercaptopropionic acid
mRNA	messenger RNA
m-SAE	meniscus-modified SAE
NA	nucleic acid
NME	nanostructured microelectrodes
NP	nanoparticle
NPP	normal pulse polarography
ocDNA	open circular DNA
ODN	oligodeoxynucleotide
OP	oscillographic polarography
POC	point-of-care
PCR	polymerase chain reaction
PMB	paramagnetic bead
PNA	peptide nucleic acid
p-SAE	polished SAE
pzc	point of zero charge
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	reporter probe
RT-PCR	reverse transcription PCR
rRNA	ribosomal RNA
SAE	solid amalgam electrode
SAM	self-assembled monolayer
scDNA	supercoiled DNA
SCE	saturated calomel electrode
SECM	scanning electrochemical microscopy
SER(R)S	surface enhanced (resonance) Raman spectroscopy
SHCP	thiolated capture probe
S/N	signal-to-noise ratio
SNP	single nucleotide polymorphism
SPR	surface plasmon resonance
ssb	single-strand break
ssDNA	single-stranded DNA
SST	single-surface technique
SSV	sphere segment void
STR	short tandem repeat
SWV	square wave voltammetry
tDNA	target DNA
TFO	triplex-forming oligonucleotide
TMB	3′,3′,5′,5′-tetramethylbenzidine

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NOTE ADDED IN PROOF

After finishing this article, a number of papers dealing with electrochemistry of NAs have been published, e.g.,[905−924] including several reviews on interesting topics, such as cyclic voltammetry of metal-based antitumor drug-DNA interactions,[916] redox indicators for DNA EC analysis,[917] electrically heated electrodes,[918] integration of amperometric detection with electrophoretic microchip devices,[919] SAMs for EC sensing,[920] and carbon paste electrodes for DNA analysis.[921] Very recently, a special issue of The Chemical Record has been published on the occasion of 90 years of Polarography. This issue included articles on the history of polarography by P. Zuman[922] and M. Heyrovsky,[923] as well as article on electrocatalysis in DNA and other biomacromolecules,[924] in addition to a number of papers from different electrochemistry fields.