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Sensitive electrochemical assays of DNA structure

Electrochemical analysis of DNA

ABSTRACT

Electrochemical methods have been used to study the structure and function of nucleic acids for more than 50 years. These approaches complement other experimental techniques, which we illustrate by using examples from studies of processes involved in the repair of DNA damage. The excellent sensitivity of the electrochemical approaches makes them good candidates for use as biosensors of a wide range of molecules and biological processes.

KEYWORDS

Electrochemistry, polarography, chronopotentiometry, DNA structure, DNA damage, DNA hairpin, DNA ligase, biosensor.

BACKGROUND

Nucleic acids are biomolecules that are hugely important for information transfer processes in all organisms, allowing them to grow and divide as needed. Thus, as DNA and various forms of RNA, these are essential molecules in all cells. Although nucleic acids are large molecules, they are relatively simple, consisting of a regular phosphodiester backbone and each type (DNA and RNA) contains 4 different bases that can come together to form base pairs (1). The combination of the ability of accessible base residues in nucleic acids to accept or deliver electrons on interaction with electrodes, as well as the regular repeating structure and highly negatively charged backbone, provides opportunities for these molecules to be analysed using electrochemical methods. However, initial experiments with DNA suggested that it was not electro active. In hindsight, pioneering electrochemical studies on DNA struggled from a lack of interest among electrochemists, who preferred simpler systems for their electrochemical studies, and it took many years for nucleic acids to become appreciated as molecules that are amenable to analysis by electrochemical approaches (2).
However, this research field has blossomed in recent years, with the requirement to improve the speed and efficiency of genome sequencing methods and for high-throughput analysis of gene expression acting as significant driving forces in the development of new technologies based on electrochemical measurements (3-6).

DEVELOPMENTS IN ELECTROCHEMICAL TECHNOLOGIES

The first electrochemical studies of DNA were performed about 50 years ago (2, 7), but the next three decades saw relatively limited research activity in this area. However, during these decades a number of important advances were made, such as the introduction of covalently bound electro active markers into DNA, the application of solid electrodes and the invention of DNA-modified electrodes. All of these are now being developed further to serve in DNA sensors. For example, electro active markers based on osmium tetroxide complexes with nitrogenous ligands were proposed at the beginning of the 1980's but were little utilized by electrochemists at that time, though they were then shown to be excellent chemical probes of DNA structure (8). Using these complexes, local structures stabilized by DNA supercoiling – such as cruciform, left-handed DNA segments and triplexes – were studied at single-nucleotide resolution both *in vitro* and in cells (9). Currently, they are also utilized in electrochemical DNA sensors (see below). Since the start of the 1990's there has been a huge increase in electrochemical analyses of nucleic acids, with these developments particularly related to progress in genomics, especially in the Human Genome Project. As the technologies have matured it has become clear that electrochemistry has much to offer those that study nucleic acids as the instrumentation is becoming ever cheaper and more portable. Furthermore, electrochemical assays are attractive to researchers because they can be "label-free" and, therefore, have relatively little influence on the system under study. In some cases, the use of specific reporter molecules offers advantages because they allow the electrochemical signals to be amplified, thus producing assays that are more sensitive. Here, the range of electrochemical techniques available for studies of nucleic acids will only be described briefly since they have been the subject of several recent reviews (10-14).
The electrochemical signals that are detected in various techniques are dependent on the nature of the nucleobases and on whether they are present in single- or double-stranded regions of the nucleic acids. Accordingly, the methods that are now available can provide independent and complementary information about the structure of nucleic acids and the molecules that they interact with. Here we will discuss how electrochemical approaches provide useful tools for detecting changes in the structure of nucleic acids, such as those that occur upon hybridization of additional bases or strands, or during DNA damage. Large differences in electrochemical responses at mercury electrodes (due to reduction of adenine and cytosine bases) have been observed between single-

and double-stranded DNAs derived from chromosomal, viral and plasmid sources (Figure 1). These differences are related to the local environment of the bases (and their primary reduction sites). In the interior of the DNA double helix they are not normally accessible to the electrode. Using mercury electrodes minor damage to DNA by chemical or physical agents is easily detectable because the affected bases then become accessible (13). By contrast, carbon electrodes (reporting on oxidation of guanine and adenine bases) show little sensitivity to the formation of DNA single-strand breaks and other local changes in the DNA duplex. Notably, electrochemistry with mercury electrodes provided early evidence of the polymorphic structure of the DNA double helix (15) and DNA pre-melting. It was not only the nature of the electrodes that played a critical role in analyses of DNA and RNA, but also the choice of electrochemical method.

For example, the first experiments with direct current polarography showed poor sensitivity in the analysis of denatured single-stranded DNA and complete inactivity with native double-stranded DNA.

Much better results were obtained using oscillographic polarography with controlled alternating current (cyclic alternating current chronopotentiometry, according to the present nomenclature). This method was replaced in the middle of the 1990's by pulse polarography and square wave voltammetry. The latter method, as well as constant current chronopotentiometry with base-line correction, is particularly useful when working with solid electrodes. The application of stripping methods to exploit the strong adsorption of nucleic acids and bound components at some electrodes greatly increases the sensitivity of electrochemical nucleic acid determinations. Using these methods it is possible to accumulate nucleic acids or their bases at the electrodes and detect them at nanomolar, picomolar and, in some cases, even sub-picomolar concentrations. When adsorptive transfer stripping (*ex situ*) methods are used in conjunction with miniaturized electrodes, a few microlitres of solution are sufficient for the analysis. Current miniaturization of electrodes will result in further decrease of the requirements for the analyte volumes.

DNA-protein interactions play a critical role in basic biological processes, such as DNA replication, transcription and DNA repair. Until recently, electrochemical analysis of proteins was limited predominantly to conjugated proteins containing non-protein redox centres. Using constant current chronopotentiometric stripping analysis it has been shown that practically all proteins produce a well-developed electrocatalytic peak (peak H). By monitoring peak H it is possible to detect peptides and proteins at nanomolar concentrations. Moreover, this peak is sensitive to changes in protein structure and its usefulness has been demonstrated, for example, in the analysis of changes in the structure of α-synuclein (important in Parkinson's disease) (16) and the wild type and mutant forms of the tumour suppressor protein p53. Peak H thus represents a new tool that is applicable to the study of DNA-protein interactions (17).

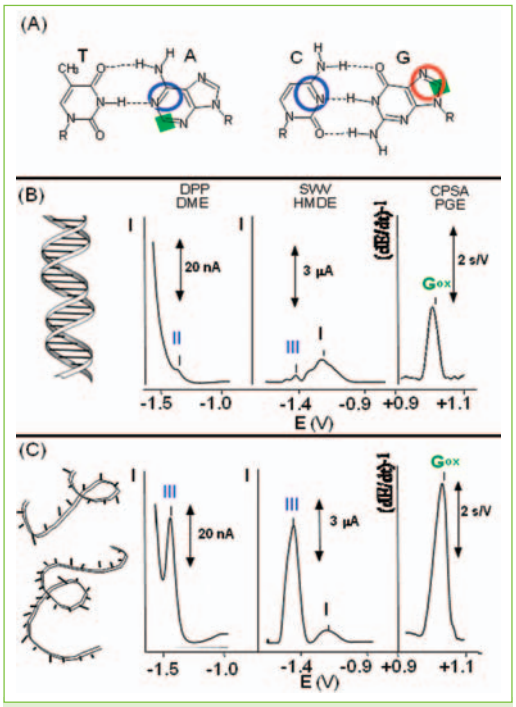

Figure1. Reduction and oxidation of single-stranded and double-stranded nucleic acids at electrodes. (A) Schematic representation of Watson-Crick base pairs and electro active groups. Primary reduction and oxidation sites are shown as observed at mercury (rectangles) and carbon electrodes (circles). (B, C) Redox signals obtained with various electrodes that analysed 100 µg/ml of native (double-stranded) (B) or 50 µg/ml of denatured ssDNA (C). Methods and electrodes used were differential pulse polarography (DPP) at the static dropping mercury electrode (DME), adsorptive stripping square-wave voltammetry (SWV) at a hanging mercury drop electrode (HMDE), and constant current chronopotentiometric stripping analysis (CPSA) at a pyrolytic graphite electrode (PGE). Roman Numerals refer to well-characterised peaks that are observed with the different methods. Adapted with permission from (12). Copyright 2001 American Chemical Society.

BIOSENSORS AND BIOSENSING TECHNIQUES DERIVED FROM ELECTROCHEMICAL ANALYSIS OF NUCLEIC ACIDS

The recent surge in interest in electrochemical analysis of nucleic acids can be mainly attributed to their use as biosensors. The major link between electrochemistry of DNA and biosensors stems from the usefulness of this technique to detect whether the two strands of DNA are fully hybridised (6, 10, 12). The element frequently used as the reporter in hybridization sensors is a single-stranded DNA (ssDNA) of defined nucleotide sequence, which acts as a "probe" (Figure 2). Upon being challenged with another ssDNA, if the target DNA has a nucleotide sequence that forms complementary base pairing with the probe, then a hybrid duplex is formed and this event can be detected electrochemically in various ways. Label-free approaches, utilizing intrinsic DNA electro activity, have proven to be particularly suitable in cases of asymmetrical distribution of an electro active base in the probe and target strands. For example, when the probe lacks

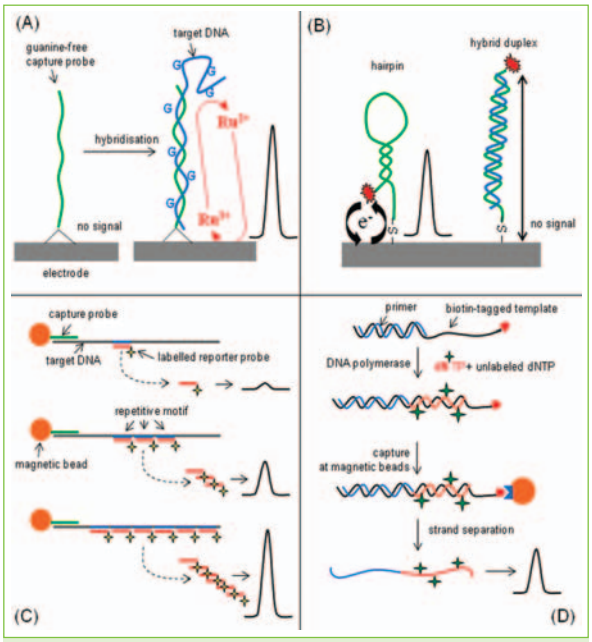

Figure 2. Examples of electrochemical biosensors and bio sensing techniques proposed for the analysis of nucleotide sequences. (A) Label-free biosensor for DNA hybridisation employing guanine-free capture probe and a soluble redox mediator to facilitate oxidation of guanines within the target DNA. (B) Electrochemical molecular beacon consisting of electrode surface-confined hairpin-forming oligonucleotide bearing redox active label on the opposite end. Conformational change upon hybridisation with target DNA makes the label "invisible" for the electrode due to increased distance. (C) Double-surface magnetic beads-based assay of a repetitive sequence length. The higher the number of repeated motifs complementary to a short labelled reporter probe, the higher the amount of the label collected per target DNA strand, which is reflected in the signal intensity. (D) Analysis of sequence-specific incorporation of nucleotides bearing electro active tags into DNA by primer extension.

guanines but the target DNA contains them, the hybridization event can be detected using the guanine signal at carbon electrodes (Figure 2A). To improve the performance of sensors working on this principle, increased electron yields were obtained with modified capture probes that used hypoxanthine bases (which pair, like guanine, with cytosine) and redox mediators were introduced that increase electron yields of oxidation of guanines within the hybridized target DNA (6). Electrochemical identification of oxidised products of guanine, such as 8-oxoguanine, has also been proposed to provide a useful sensor of oxidative stress (18).

From initial studies, the potential usefulness of DNA biosensors was abundantly clear and approaches were developed to improve the sensitivity of the techniques. Improvements included the use of DNA intercalators or groove binders that act as redox indicators of the presence of double-stranded DNA (dsDNA), or attachment of covalent tags to either the target DNA or signalling probes. Covalent modification (labelling) of DNA with redox-active moieties is a particularly promising approach owing to unambiguous discrimination between the probe and target strands. Labelled DNA can be prepared via solid-phase oligonucleotide synthesis, chemical modification of natural DNA (e.g., by the osmium tetroxide reagent mentioned above (8)) or enzymatic incorporation of labelled nucleotides by primer extension (19, 20). The use of redox indicators or covalently bound labels has enabled electrochemical detection of point mutations in hybridisation or primer-extension experiments, due to the formation of single base mismatches or to incorporation of a specific label dictated by the given single nucleotide polymorphism, respectively. Redox markers have been applied in various electrochemical devices such as those employing DNA hairpin-based molecular beacons (Figure 2B) (11, 21) or systems using DNA-mediated charge transfer (10). In this general context it is noteworthy that a variety of sensitive and selective biosensors, for small molecules as well as proteins, have been devised that utilise DNA (or RNA) aptamers anchored to a suitable electrode, to complex with the analyte of interest (22). The coupling of enzymes, such as horseradish peroxidase or bilirubin oxidase, with a redox polymer and carbon electrode has also been used to assay for nucleic acids (23, 24). Amplification of the signal by the enzyme provides a sensitive assay that is specific for a particular RNA or DNA due to the use of oligonucleotides that are complementary to the sequence under test. Currently, a number of approaches have been developed that use electrochemical approaches in DNA hybridization sensors (3, 4, 6). During the current decade, the classical concept of an electrochemical DNA hybridization sensor consisting of an electrode with a capture probe immobilized at its surface has been complemented by alternative techniques employing magnetic beads as the surface on which the DNA hybridization is conducted (Figure 2C). Then, target DNA, signalling probe or other indicator molecules captured or generated at the surface of the beads can be determined electrochemically. This "double-surface" strategy has been applied in a variety of ways to detect DNA hybridization, the incorporation of labelled nucleotides, and DNA damage as well as for probing DNA-protein interactions (reviewed in 4-6, 10-12, 21). Examples of the usefulness of electrochemical methods in studies of DNA structure is provided by assays of DNA ligases. These are enzymes that join breaks in the backbone of DNA; the bacterial proteins are potential targets for novel antibiotics (25). In attempts to improve assays of these enzymes, researchers have turned to electrochemical studies of DNA molecules. These studies have used ferrocene as a reporter group, linked to various types of hairpin DNA molecules (Figures 2B and Figure 3) (11, 21, 25). Interestingly, these various assays have also taken advantage of the potential to use fluorescence detection instead of electrochemical approaches (26, 27).

ASSAYS DETECTING NUCLEIC ACID STRUCTURE AND DNA DAMAGE

It is clear that the signals detected by various electrochemical techniques are sensitive to the nature of the nucleobases and whether they are present in single- or double-stranded

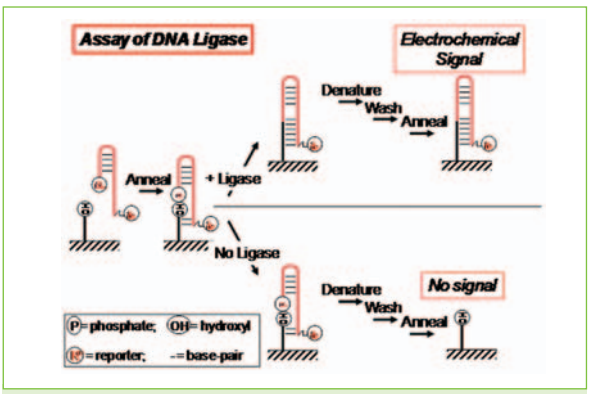

Figure 3. Analysis of DNA ligase activity using immobilized DNA hairpins. Reporter molecules, such as ferrocene, provide an electrochemical signal that allows detection of one end of the hairpin. The presence of DNA ligase in the assay leads to retention of the electrochemical signal during the assay. Note that related experiments can be performed to assay nuclease activity: incubation of fully-formed hairpins (i.e. no break in the DNA backbone) with the nuclease will lead to cleavage of the DNA backbone and loss of the electrochemical signal (27).

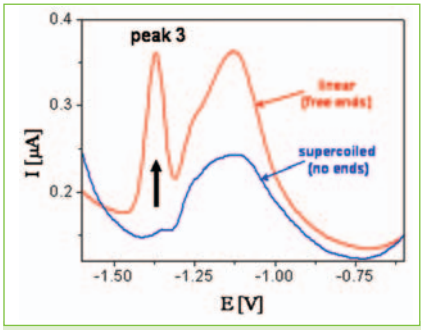

Figure 4. The electrochemical behaviour of DNA allows discrimination between molecules that contain or lack free ends, as indicated by "peak 3". Such experiments provide a sensitive electrochemical assay of the activities of enzymes that alter the backbone structure of DNA.

regions of the nucleic acids. They also depend on whether the DNA adsorbed at the electrode surface can undergo a structural transition connected with a change in nucleobase accessibility (e.g. see Figure 2). Accordingly, the electrochemical signals from mercury and some types of amalgam electrodes provide useful tools to detect structural changes that are associated with DNA damage. Besides thermal sub-denaturational (pre-melting) effects (15), experiments with supercoiled plasmid DNA revealed the sensitivity of voltammetric responses at the mercury electrode towards negative superhelix-induced structural transitions within circular duplex DNA (28). In addition, electrochemical behaviour of DNA at the mercury-based electrodes is strongly influenced by its backbone structure, allowing a perfect discrimination between DNA molecules containing or lacking free ends (13, 29, 30). Under certain conditions, the DNA double helix undergoes electrode potential-induced surface denaturation around its free ends, thus giving rise to extensive regions of ssDNA. These regions produce a specific voltammetric signal that is not observed with covalently closed circular DNAs whose denaturation is prevented for topological reasons (Figure 4). This variation in electrochemical behaviour has been utilized for sensitive detection of breakage to the DNA sugar-phosphate backbone, as occurs from reaction with some enzymes and chemical agents, such as hydroxyl radicals. The electrochemical technique was used to monitor nicking of supercoiled plasmid DNA with deoxyribonuclease I (31) as well as by DNA repair nucleases recognizing specific nucleobase lesions, such as T4 endonuclease V (cleaving DNA containing pyrimidine dimer photoproducts) or exonuclease III (cleaving at abasic sites) (29). Recently the same principle has been used for monitoring of the reverse process, i.e. the repair of strand breaks by the action of DNA ligases, including distinction between ligatable and unligatable breaks in circular plasmid DNA (30). Other types of electrodes applied to electrochemical analysis of DNA (including the popular carbon electrodes) do not provide such a high sensitivity to DNA structure changes in their label-free applications. To overcome this limitation, several methods combining DNA structure-sensitive redox markers (such as osmium tetroxide complexes that selectively modify unpaired thymine residues (32)) or indicators (metallointercalators binding preferentially to intact dsDNA (33)) with voltammetric measurements at carbon electrodes have been proposed as efficient assays and biosensors for DNA damage. Electrochemical analysis of DNA damage has also been proposed to provide useful assays for the screening of the toxicity of chemicals (drugs) and oxidative stress (18, 34-37).

CONCLUSIONS

For 50 years, electrochemical studies have provided important information about the structure of DNA and processes acting upon it. However, it is since the start of the 1990's that this research field has really blossomed, being driven forward by developments in high-throughput analyses of genome sequences and the genes that they express. Electrochemistry has much to offer such studies because the instrumentation required is relatively cheap and portable. With appropriate choice of the detection signal, additional advantages can include the use of "label-free" methods, which minimise perturbation of the system under study, or the inclusion of specific reporter molecules that maximise assay sensitivity. As illustrated by the hybridisation technologies, electrochemical methods are useful for detecting alterations to nucleic acid structure, as may occur during binding of specific drugs or proteins to DNA. Consequently, these approaches have great potential in pharmaceutical research. Further improvements in the technology are required before electrochemical DNA biosensors reach their full promise, but the great possibilities that exist suggest that this field is set to be a major focus of ongoing basic and applied research.

ACKNOWLEDGEMENTS

The idea for this review originated from development of the programme for the symposium "DNA-based electrochemical devices and biosensors" that is being held at the 42nd IUPAC Congress in Glasgow, UK, from August 2-7, 2009. The Congress is being organised by the Royal Society of Chemistry and we gratefully acknowledge their support in developing the symposium. EP and MF acknowledge support from the Academy of Sciences of the Czech Republic for institutional research plans AV0Z50040507 and AV0Z50040702.

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