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Mercury and Mercury Electrodes in the Electrochemistry of Biopolymers: Are They Really Inevitable in the Decade of Non-Mercury Sensors? (A Consideration)

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Abstract: In this article, electrochemical properties of nucleic acids and proteins at mercury electrodes are briefly reviewed. We focus on structure sensitive DNA and protein sensing and/or techniques based on the utilization of catalytic hydrogen evolution i.e., analyses that are inherently connected with the mercury electrodes. Advantages of these approaches are briefly summarized and discussed towards answering the title question regarding necessity of mercury electrodes in biopolymer electroanalysis.

Keywords: Mercury electrodes; Nucleic acids; Proteins; Structure; Interactions; DNA-damage catalytic hydrogen evolution; Denaturation; Aggregation; Review

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Introduction

The beginnings of electrochemistry of nucleic acids [1] and proteins [2,3], similarly as the beginnings of electrochemistry as such, were tightly connected with mercury electrodes. Specific features of the mercury electrodes (such as considerably high hydrogen overvoltage allowing measurements in aqueous media at very negative potentials, atomically flat surface offering efficient adsorption of the biopolymers reflecting their structures, chemisorption of sulfhydryl groups and catalytic hydrogen evolution accompanying redox processes undergone by certain intrinsic moieties or extrinsic groups attached to the biopolymers), make the mercury electrodes well suited for specific purposes in the biopolymer sensing.

Although various types of solid, mostly non-mercury electrodes are preferred in current electroanalytical chemistry, the mercury-based electrodes still remain superior in many respects. The broad field of nucleic acids and protein electrochemistry has been reviewed thoroughly (*e.g.,* [1,4]); in this paper, we focus on the behavior of biopolymers at mercury electrodes and related applications.

Structure-Sensitive Electrochemistry of Nucleic Acids at Mercury Electrodes

Electrochemical responses of nucleic acids at the mercury electrodes are strongly influenced by the NA structure. Cytosine (C) and adenine (A) residues are, when protonated, irreversibly reduced at potentials around -1.5 V vs. Ag|AgCl|3M KCl (to which are related all potentials mentioned in this paper). Guanine (G) is also reduced at the mercury and amalgam electrodes but its reduction requires application of more negative potentials (≤-1.6 V) and cannot be observed directly due to strong currents of background electrolyte decomposition in the same potential region (range). Since the primary reduction sites in both C and A, double bonds N3=C4 (in C) or N1=C6 (in A), are hidden in the interior of DNA double helix, in intact native (double-stranded, ds) DNA these sites are prevented from being reduced at the electrode. On the contrary, in denatured (single-stranded, ss) DNA the nucleobases are freely accessible for communication with the electrode and the ssDNA produces well developed cathodic peak CA (Fig. 1A) corresponding to reduction of both C and A.

It became clear soon after the discovery of the DNA electrochemical activity that not only differentiation between ss and ds DNA (representing two rather extreme states), but also monitoring of much more suble changes in DNA structure is feasible using polarographic (with the dropping mercury electrode, DME) or voltammetric (with the hanging mercury drop electrode, HMDE) measurements. Reducibility of C and A residues reflects distortions of the DNA double helix, as well as transient base pair openings in dependence to temperature changes in the pre-melting region (i.e., before the complementary DNA strands are separated) [5]. Susceptibility of DNA to undergoing such transitions depends on the base composition and nucleotide sequence, and in this respect observations made using differential pulse polarography brought the first evidence that structural polymorphy of the dsDNA. In this respect, the polarographic measurements of DNA were ahead of their time and conclusions made on their basis were not generally accepted in the 1960's and even 1970's unless DNA structure polymorphy was discovered using methods better acceptable by biologists.

Structure effects on the DNA responses at the mercury electrodes are closely related to DNA adsorption at mercury, in which the hydrophobic bases – when accessible to the environment – play an important role [1,6]. In weakly alkaline media the cathodic reduction does not take place, but specific tensammetric (capacitive) current signals can be obtained which are sensitively responding to the DNA structure as well (Fig. 1B). Intact B-form dsDNA, in which bases (base pairs) are not exposed to the environment, produces only one capacitive peak 1 which has been ascribed to desorption (or reorientation) processes of DNA segments adsorbed via sugar-phosphate backbone due to electrostatic

repulsion from the negatively charged mercury surface. When the dsDNA contains conformational "defects" such as bent or untwisted (but still base-paired) regions, peak 2 is observed and its intensity reflects extent of the conformational distortion(s) [6,7]. Appearance of the peak 2 has been discussed in terms of desorption/reorientation of DNA regions in which edges of base pairs are partly exposed. Such regions are adsorbed more firmly than those adsorbed exclusively via the DNA backbone, and their desorption requires higher activation energy (more negative potential). In base-unpaired regions of DNA (or i ss DNA) the bases are freely accessible, firmly adsorbed at the surface, and desorption of such DNA regions takes place at most negative potentials, giving rise to the peak 3. Taken together, measurements of capacitive responses at mercury electrodes represents a simple way to label-free probing various DNA structural motifs and structural transitions, including those related to DNA interactions with small molecules.

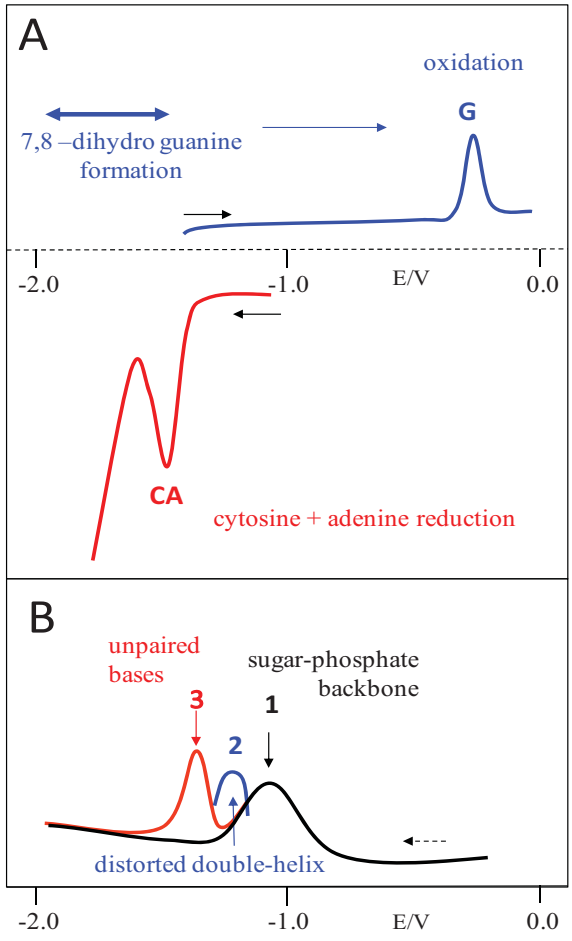

Fig. 1: *Overview of faradaic (**A**) and tensammetric (**B**) DNA responses at mercury electrodes.*

Polarographic techniques (with DME) using small potential excursions during the drop lifetime revealed qualitative difference between responses of ds and ssDNAs. The dsDNA yielded a small signal (in DPP denoted as peak II) at a potential less negative than much higher signal of the ssDNA (peak III) [5]. On the other hand, when voltammetric techniques (with HMDE) were used, the ssDNA-specific signals (faradaic or tensammetric) were detected with dsDNA, suggesting surface denaturation of the DNA double helix at the electrode. It has been established that dsDNA unwinding takes place at the HMDE within a relatively narrow (in neutral medium) potential region around -1.2 V vs. SCE ("region U") [6,8]. When dsDNA is adsorbed at the electrode and the potential is slowly scanned from positive to negative values, certain portion of the double helix is unwound before potentials corresponding to the ssDNA-specific signals (such as tensammetric peak 3) are reached (Fig. 7). Notably, this unwinding process is feasible only with DNA molecules containing free ends (strand breaks) which has been utilized in detecting DNA damage by various physical or chemical agents [6], including those generated in situ by electrochemical processes [9,10], as well as DNA cleavage or ligation by enzymes [11,12].

Electrochemistry and Electrocatalytic Features of Proteins at Mercury Electrodes

The history of protein electrochemistry started as early as in the 1920s owing to the pioneering work by J. Heyrovsky, R. Brdicka and others who observed for the first time electrocatalytic properties of proteins taking place under certain conditions at the mercury electrodes [13,14]. Similarly as for the nucleic acids, intrinsic electroactivity of proteins is connected with the presence of electroactive moieties in their molecules. Electrochemical behavior of proteins at the mercury electrodes is strongly influenced by the presence or absence of cysteine. Thiol group of this amino acid exhibits a strong affinity to mercury, forming stable mercury thiolate complexes [15,16]. Detection of these species is possible via reduction of the sulfur-mercury bond. In addition, signals due to disulfide reduction (in cystine, a dimeric disulfide-bridged form of cysteine typically present in extracellular peptides or proteins) have been reported. In the presence of cobalt (or some other transition metal) ions, signals due to catalytic hydrogen evolution can be detected with cysteine-containing peptides and proteins ("Brdicka catalytic response", BCR [14], reviewed in [2,3,17]). Usefulness of the BCR in bioanalysis, especially in detection of cysteine-rich species such as metallothioneins [18] or phytochelatins [19], has been demonstrated. Last but not least, practically all peptides or proteins (not only the cysteine-containing ones) can produce signals due to electrocatalytic hydrogen evolution at the mercury-based electrodes in the absence of the transition metal ions. This "presodium catalysis" was for the first time reported by J. Heyrovsky and J. Babicka in the late 1920s [13] but its potentialities for protein studies have emerged in connection with application of constant current chronopotentiometric stripping (CPS) and discovery of the "peak H" [3,20].

In addition, growing amount of experimental data demonstrate that electrochemical techniques are well suited for studies of changes in the protein structure involving their unfolding (denaturation) [21], aggregation [22,23], redox (thiol-disulfide) "switching" [24,25] or interactions with specific ligands [26].

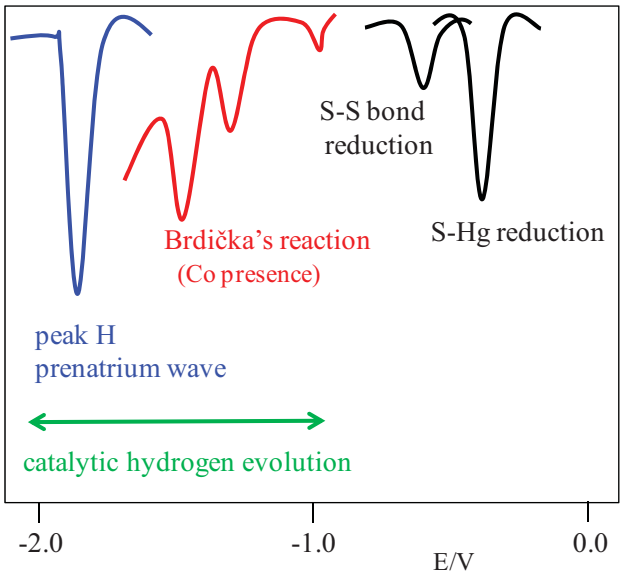

Fig. 2: *Overview of electrochemical and electrocytalytic responses of proteins at mercury electrodes.*

Catalytic Signals of Chemically Modified DNA

One of important advantages of mercury, compared to other metallic or non-metalic electrode material, is a considerably high hydrogen overvoltage allowing measurements in protic (including aqueous) media at relatively highly negative potentials. At the same time, this feature makes the mercury electrodes well suited for studying and utilizing processes coupled to catalytic hydrogen evolution. While electrocatalytic activity of proteins (see above) is rendered them by their intrinsic components, natural nucleic acids were not observed to catalyze hydrogen evolution connected with appearance of analytically useful current signals. On the other hand, CHE was shown to be useful for the determination of DNA modification with certain moieties, namely those containing transition metals. Osmium tetroxide complexes with tertiary amines were probably the first species used for DNA labeling with electrochemically active extra groups [27]. These species react covalently with pyrimidine residues, typically showing a high selectivity for thymine bases in single stranded DNA [28]. The resulting adducts show well pronounced electrochemistry related to redox transitions of the central osmium atom which can be observed at mercury as well as non-mercury (carbon or gold) electrodes [28-31].

In addition, a strong CHE takes place at the mercury electrodes, accompanying the last reduction step of osmium [29] (Fig. 3A, peak 3; compare intensity of this signal produced by globally modified ssDNA with peak CA yielded by C+A residues in unmodified DNA at the same concentration). Using this high-electron-yield catalytic signal, considerably lower detection limits of the modified nucleic acid determination have been attained [32], compared to detection limits reached for the faradaic signals involving one or two electrons per osmium moiety. Similar CHE effects have also been demonstrated for oxoosmium modified proteins and saccharides, including sugar moieties in ribosides and ribonucleic acids [33,34].

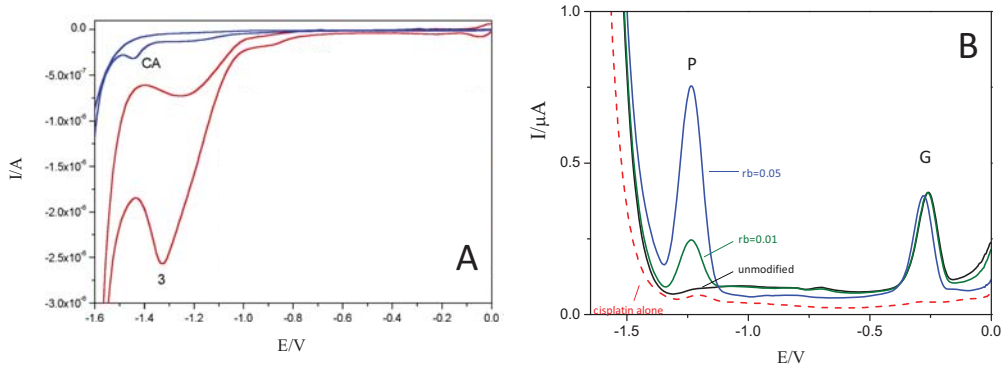

Fig. 3: *Examples of catalytic hydrogen-evolution responses of DNA modified with osmium (**A**, peak 3) or platinum (**B**, peak P) moieties.* **Legend:** (**A**) cyclic voltammetry, initial potential 0V, switching potential -1.85 V, scan rate 0.2 V s^{-1}; blue - unmodified DNA, red - the same DNA globally modified with osmium tetroxide, 2,2'-bipyridine. (**B**) square wave voltammetry, intial potentia1 -1.85 V, final potential 0 V, frequency 200 Hz, amplitude 50 mV, potential step 5 mV; black – unmodified DNA, green and blue – cisplatinated, rb (Pt per nucleotide) 0.01 and 0.05, respectively, red – cisplatin in the absence of DNA.

Similarly, DNA modified with an antineoplastic drug *Cisplatin* has recently been shown to exhibit analytically useful CHE [35]. Using anodic stripping square wave voltammetry with the HMDE, a symmetric peak (Fig. 3B, peak P) has been obtained, the intensity of which was linearly responding to the number of cisplatin adducts per nucleotide (in Fig. 3 denoted as rb). From a comparison of the peak intensities in Figure 3B one can see the catalytic peak P steeply increasing with the DNA platination level, while the DNA peak G remains practically unchanged. Note that one platinum adduct per 100 - 20 nucleotides produces CHE signals the intensity of which is in the same order of magnitude as peak G yielded by one guanine per 4 nucleotides on average. It can be seen that such sensitivity of DNA-bound platinum determination meets the requirements for monitoring the DNA modification at the levels relevant for typical studies of the influence of DNA cisplatination on its molecular recognition features [35].

Biopolymer Analysis with Other Electrodes

Recent trends in (bio)analytical electrochemistry follow the concept of "green chemistry" oriented towards minimizing the usage of poisonous chemicals and materials. Toxicity of mercury in its metallic and other states, related to utilization of mercury electrodes in electroanalysis and in its entirety, is discussed thoroughly within this issue – in the contribution by Navrátil et al. Herein, our attention is focused on possible alternatives to the mercury electrodes and, mainly, with respect to their applicability in the above-mentioned types of analyses.

Perhaps the most popular non-mercury electrodes in biopolymer analysis are various types of *carbon electrodes*, including glassy carbon, pyrolytic graphite in both basal plane and edge orientation, carbon pastes, boron doped diamond, disposable screen printed and pencil graphite electrodes as well any of these electrodes modified with conductive polymers of "nanoobjects" such as metallic nanoparticles, carbon nanotubes etc. Evaluation of these materials regarding their specific features is out of the scope of this article; in general, any of them can in principle be used for label free analysis of both nucleic acids (via electrochemical oxidation of nucleobases, particularly purines [1,6]) and proteins (via electrooxidation of side groups of tyrosine and/or tryptophan [3]). The respective current signals exhibit certain level of sensitivity to the biopolymer structure; however, compared to the above described effects observed with the mercury electrodes, changes in the signal intensities due to the biopolymer structural transitions are poorly pronounced. For DNA this is caused by relatively facile purine oxidation in dsDNA on one hand and absence of extensive double helix unwinding at carbon, under conditions compatible with measuring the oxidation responses, on the other. Thus, formation of one or several strand breaks per plasmid DNA molecule, well detectable with the mercury electrodes, does not change significantly the behavior of DNA at carbon electrodes [6].

Better results are obtained when chemical modification to the DNA is detected, especially when this modification rely in introducing new electroactive moieties giving rise to signals not produced by unmodified DNA. Label- or indicator based approaches [36] using DNA as biorecognition element belong to the most frequent topics in current DNA electrochemistry using carbon electrodes. Nevertheless, in contrast to the mercury electrodes, carbon electrodes are not well suited for measuring the CHE. The same applies to *gold electrodes*, representing the most popular platform for (not only electrochemical) biosensors construction. Here, direct electrochemistry of unmodified DNA or non-conjugated proteins has seldom been applied. Instead, nucleic acid probes are immobilized on gold via thiol linker to create biorecognition layer usually for DNA hybridization experiments, and the proper electrochemical detection of the hybridization events is based on utilization of various redox markers.

Non-mercury materials applied as nontoxic mercury surrogates in analyses traditionally connected with the mercury electrodes include *bismuth* and more recently also *antimony* ones [37]. Albeit bismuth electrodes have found relatively broad application in anodic stripping of metals and in

analysis of some organic species (such as nitrocompounds), attempts to apply them in DNA analysis have not been successful. It appears that ineffective adsorption of DNA at the bismuth surface was the main reason why we failed to detect any DNA reponse at bismuth films or pastes, as even DNA globally modified with osmium did not show any signal within the bismuth eletrode working window. Notably, mixed carbon/bismuth (powder) paste electrodes prepared for different carbon/bismuth ratios showed linear decrease of the osmium signal with increasing bismuth content (P. Horáková and M. Fojta, unpublished). To our knowledge, to date, there have been no attempts to apply antimony electrodes in biopolymer electrochemistry.

In contrast to all above mentioned alternatives, *amalgam electrodes*, especially various modifications of solid silver amalgam, have been demonstrated to be powerful substitutes for the classical mercury ones not only in the determination of inorganic metal ions and relatively simple organic compounds, but also in applications oriented towards biopolymer sensing. Label-free structure selective analysis of DNA has successfully been performed with solid silver amalgam electrode modified with mercury meniscus (m-AgSAE) as well as with a electrolytically deposited mercury film (MF-AgSAE), while polished p-AgSAE showed a weaker DNA adsorption and less well pronounced (but still detectable) DNA tensammetric and faradaic DNA responses [38]. Again, these observations underline the importance of DNA adsorption on the smooth mercury surface for obtaining a good discrimination among different DNA structures. CHE accompanying redox processes of DNA-bound osmium [39,40] or platinum [35] moieties was detected at the mercury-modified amalgam electrodes as well; notably, measurements made with the osmium DNA adducts revealed a critical minimum thickness of the mercury film ti obtain a well developed catalytic signal [40]. No CHE was observed for p-AgSAE lacking any mercury layer on its surface. In measurements with cysteine containing peptides, m-AgSAE and MF-AgSAE showed Brdicka catalytic responses similar to those measured at the HMDE, while the p-AgSAE showed surprisingly higher sensitivity but different signal patterns [41]. Peak H allowing discrimination between native and denatured proteins was obtained on the m-AgSAE as well as on electrolytically amalgamated silver substrates [42].

So, Do We Need Mercury for Biopolymer Analysis?

To answer reasonably the title question, one should consider what he/she would expect from an electroanalytical technique. There are lots of applications in which extremely high senstivities towards amounts and/or structural features of the biopolymer analyte are not inherently needed. For example, many sequence specific DNA assays involve polymerase chain reaction amplification of a specific piece of DNA so that the DNA amount is not limiting. These types od assays do not involve probing of subtle changes in DNA conformation, but are applied to indicate presence or absence of a DNA strand of specific nucleotide sequence (often encoded by an electroactive label).

In the field of DNA damage sensing there are also instances not requiring extreme sensitivities, e.g. when potential genotoxicity of a compound is tested or long incubation times are affordable to accumulate DNA lesions prior to the measurements. However, since the latter approaches usually employ decrease of guanine oxidation signal at carbon for quantification of the DNA damage, they inherently suffer from the lack of both sensitivity (small decrease of a large initial signal is to be detected) and selectivity (signal decrease may *e.g.* be caused by electrode fouling due to surface-active species). In the field of analysis of non-conjugated proteins, it is even more difficult to find alternatives to the structure-selective catalytic signals at mercury or highly selective detection of cysteine-rich peptides and proteins, such as metallothioneins and phytochelatins using the BCR.

Hence, the title question can be paraphrased: "*Do we need simple, mostly label free electrochemical assays sensitive to structural changes of biopolymers which are related to DNA damage, DNA interactions with potentially genotoxic species such as intercalators? Do we need a simple way to determination of low levels of DNA modification with anticancer drugs via electrocatalysis? Do we need techniques capable of detection small changes in protein conformation or early stages of protein aggregation? Do we need to monitor response of plant cells to heavy metal stress via simple electrochemical assay of phytochelatin accumulation?*" If so, the mercury electrodes or their surrogates (represented by the amalgam electrodes) are inevitable; otherwise, one would have to acquiesce with less attractive experimentation at considerably lower sensitivity and selectivity.

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