Determination of DNA Bases Using Electrochemistry: A Discovery-Based Experiment

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Abstract: A discovery-based experiment is presented for use in undergraduate analytical and biochemistry courses. The experiment uses electrochemical techniques (e.g., cyclic, linear-sweep, and/or square-wave voltammetry) to detect the presence of DNA bases in solution. Working individually or in teams, students must develop a method for the detection of adenine(A), guanine (G), cytosine (C) and thymine (T) in aqueous samples. They are given only topical information about their project and must research and plan the analyses, learn the instrumental methods to be used, and prepare an experimental protocol that will be "validated" by another individual/team during a subsequent laboratory. Goals of this approach include introducing students to various electrochemical techniques and having them research how these techniques are being used to determine and study biologically relevant analytes. Another goal is to place students in the position of being scientists and having to make decisions and recommendations. Each step of the analytical process must be carefully considered and its significance assessed because there are no "recipes" to follow as they develop their methods and make comparisons between different electrochemical techniques for the determination of analytes.

Introduction

Guanine (G), adenine (A), cytosine (A), and thymine (T) (Figure 1), are important components of DNA (deoxyribonucleic acid). Measuring concentrations of these bases or their ratios in DNA is an active and important area of research. In fact, the in vivo oxidation of DNA causes cell damage and plays an important role in mutagenesis, carcinogenesis, and has been proposed to be a major contributor to ageing and age-related diseases. Therefore, the measurement of elevated levels of these bases, and especially their oxidation products, could be indicative of certain diseases [1, 2].

Many chromatographic or electrophoretic methods coupled with spectroscopic and electrochemical detection have been developed for the detection and quantification of DNA bases in nucleic acids [1, 3]. In addition, voltammetric techniques are also being actively explored. For example, several methods for the determination of guanine and adenine upon electrochemical oxidation or reduction at various electrodes (e.g., glassy carbon, carbon paste, and chemically modified electrodes) have been reported [1, 2, 4–6].

It is well-known that electrochemical oxidation in DNA can occur at each of the four bases and that guanine oxidizes at the lowest potential (i.e., can suffer easiest oxidative damage). Recently, it has been suggested that genes in animal genomes are protected against in vivo oxidation (which may lead to aging and mutation of cells) by electrochemically reducing long-base sequences [7, 8]. In essence, these more reducing long-base sequences (e.g., G or GC rich domains) would be sacrificially oxidized, resulting in a form of "cathodic protection" for genes. Although this hypothesis has yet to be proven, it can be used to introduce undergraduate students to an active area of current chemical and biochemical research as well as various voltammetric techniques.

With the commercial availability of relatively inexpensive, computer-controlled potentiostats a wide range of electrochemical techniques are now readily accessible. To date, two have been used to study the DNA bases discussed in this paper: cyclic and square wave voltammetry. Cyclic voltammetry (CV) is by far the most widely used electrochemical method and many examples suitable for undergraduate laboratories have been published [9]. The theory of cyclic voltammetry is well established and relatively straightforward descriptions of this technique can be found in electroanalytical texts [10, 11] and papers published in the chemical education literature [12]. Square-wave voltammetry (SWV) refers to a group of techniques that employ various waveforms to increase sensitivity and aid in background suppression. A comprehensive review on square wave methods is available [13] as are introductions in electroanlytical texts [14, 15]. Readers who are unfamiliar with these techniques are referred to these references for more information.

Owing to the widespread use of electrochemical techniques in both fundamental and applied studies and the importance of introducing students during their undergraduate experience to active areas of chemical and biochemical research, we have developed a discovery-based laboratory for the electrochemical determination of DNA bases in aqueous solution. It is suitable for undergraduate analytical and biochemistry laboratories.

Experimental

Reagents. A list of possible reagents follows. Depending on student creativity, group choices, and materials on-hand, actual reagents may vary from those listed. Guanine, adenine, thymine, and Cytosine were from Sigma (Biochemical Grade), potassium phosphate monobasic, trizma hydrochloride (Tris), and sulfuric acid (Caution: sulfuric acid is corrosive and should be handled with adequate protection) were from Fisher. Unless otherwise indicated, deionized

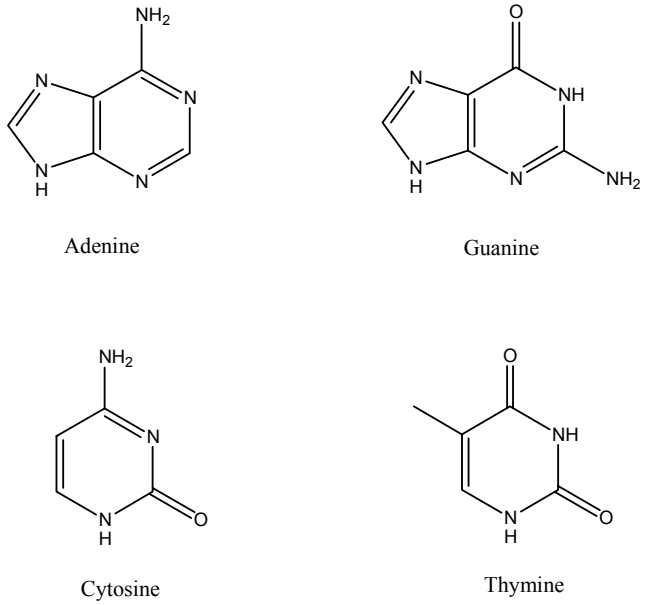

Figure 1. Structures of DNA bases.

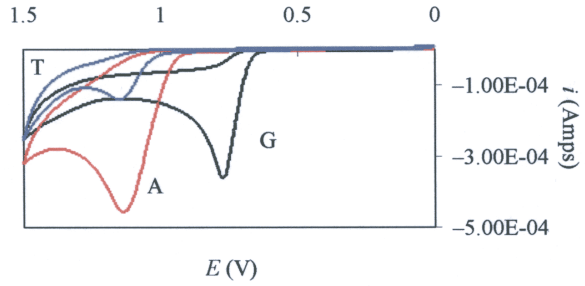

Figure 2. Cyclic voltammograms of 0.1 mM solutions of guanine (black), adenine (red), and thymine (blue) in 0.2 M KH_2PO_4 at a glassy-carbon electrode, pH = 2.75.

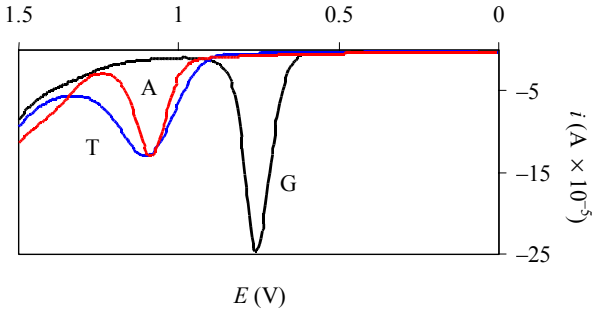

Figure 3. Square-wave voltammograms of 0.1 mM solutions of guanine (black), adenine (red), and thymine (blue) in 0.2 M KH_2PO_4 at a glassy-carbon electrode, pH = 2.75.

water passed through a Barnstead/Thermolyne triple filtration system was used in all experiments.

Solutions. One of the first challenges in this exercise is to dissolve the bases in aqueous solution. Students are encouraged to make up concentrated stock solutions from which aliquots can be taken for working solutions. A quick perusal of the Merck Index [16] shows that the solubility of the analytes in purely aqueous solution is very low. For example, the listing for guanine states "Practically insoluble in water, alcohol, ether; sol. in acidulated water" [16]. Various methods have been used to dissolve the bases, including the addition of small amounts of organic solvents (e.g., acetonitrile) to the solid base followed by dilution with water and the acidification of the solution. In the latter method, dilute sulfuric acid (or other mineral acid) is usually added to a stirred suspension of the base in deionized water. After 15 to 30 min of mixing at pH ~ 1, the bases dissolve and the pH can be raised to around 2.75. At higher pHs the bases reprecipitate.

Instrumentation. Electrochemistry experiments employed a CH Instruments Model 620 or 660 Electrochemical Analyzer and a conventional three-electrode configuration. An electrochemical cell was constructed of a 100-mL beaker and a machined Teflon cover (Bioanalytical Systems Inc.). The cover contained openings for three electrodes. Enough solution (~50mL) was placed in the cell to immerse a working electrode (5-mm diameter), a platinum wire counter electrode and a saturated Ag/AgCl reference electrode (Eo = 0.199 V versus NHE) [17]. Working electrodes containing platinum, gold or glassy-carbon disks (~5 mm) imbedded in Teflon were available for student use. Working electrodes were manually cleaned prior to and after each can by mechanical polishing on a felt pad with an aqueous slurry of 0.5mM alumina. The electrode was then rinsed with deionized water, immersed in concentrated nitric acid (~5 sec), rinsed a second time, and then placed in a small beaker containing ethanol, followed by sonication for approximately 5 min.

Results and Discussion

Typical cyclic voltammograms for guanine, adenine, and thymine using a potassium phosphate buffer system are shown in Figure 2. Square-wave voltammograms for these three bases are shown in Figure 3. In each case an electrochemically irreversible oxidation is observed at positive potentials, indicating the formation of reaction products upon oxidation {i.e., an EC reaction [18] (electron transfer followed by a chemical reaction)} or an oxygen transfer mechanism [19]. For example, 7,8-dihydro-8-oxoguanine (commonly referred to as 8-oxoguanine) has been identified as the major product of guanine oxidation [20]. This compound exists in human tissues as a product of DNA oxidation via normal metabolic pathways; however, it is found in higher concentrations in cancerous tissues as well as in the human tissues of smokers; therefore, 8-oxoguanine has been proposed as a urine biomarker for DNA oxidative lesions [20].

Although students are assigned cytosine as one of the DNA bases to test, no student group has yet been able to observe its oxidative wave in aqueous solution. The available potential range in water is simply too narrow because the oxidation of water obscures the cytosine wave in aqueous solution. The potential range that is available in aqueous solution also depends on the electrode material and electrolyte. Of the electrodes provided (i.e., platinum and carbon), students quickly realize that glassy carbon has by far the widest oxidative window (~ 0 to +1.5 V [18]). Because platinum is an excellent catalyst for water oxidation, the potential range for platinum (~+0.8 to –0.8 in pH 7 buffer [18]) does not facilitate the detection of these analytes. Figures 2 and 3 were generated in aqueous phosophate buffer solution (0.2 M; pH ~ 2.75). Similar results have been obtained in dilute (~1M) sulfuric acid and tris buffers. Student groups are encouraged to explore other solvents (e.g., acetonitrile) to expand the potential window but this is often limited by time constraints.

Typical square-wave voltammograms for a mixture of guanine and adenine are shown in Figure 4. The oxidative waves of G and A are clearly resolved in the timeframe of the voltammetric experiments; however, no group has yet been able to resolve the overlapping oxidative waves of adenine and thymine (voltammograms not shown). A square-wave voltammogram for a mixture of G, A, and T is shown in Figure

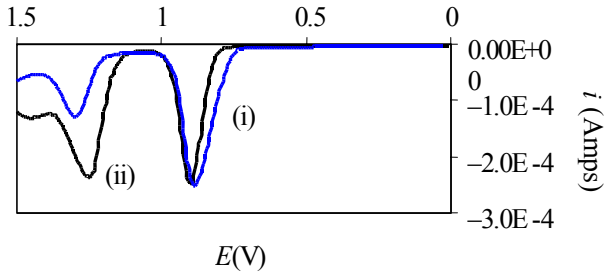

Figure 4. Square-wave voltammograms of mixtures of (i) guanine and adenine (black) and (ii) guanine and thymine (blue), 0.1 mM base in 0.2 M KH_2PO_4 at a glassy-carbon electrode. pH = 2.75.

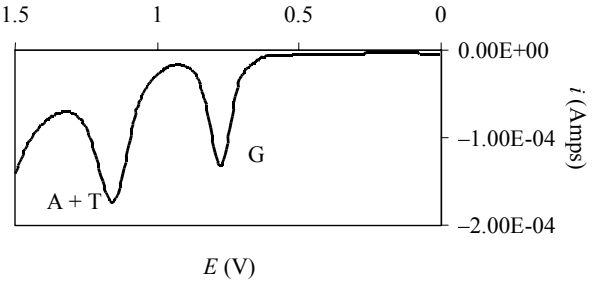

Figure 5. Square-wave voltammogram of a mixture of G, A, and T, 0.1 mM base in 0.2 M KH_2PO_4 at a glassy-carbon electrode, pH = 2.75.

5. Again, an oxidative wave for guanine is clearly visible and separate from subsequent oxidations; however, repeated attempts at varying scan rate, electrode material, and voltammetric method do not result in resolution of the A and T oxidation waves. This often-times "exercise in frustration" is a good chance to emphasize to students that while a method may be excellent for one analysis (e.g., measurement of guanine in aqueous solution), it may not be suitable for other analyses (e.g., solution containing both adenine and thymine). It should be mentioned that the potential at which a functional group will oxidize will not change with scan rate, voltammetric method, or electrode material; however, these are variables students can easily control and, although this may seem futile, it can lead to interesting discussions both during and after the exercise is completed.

Timeline of Laboratory. The "Determination of DNA bases using Electrochemistry" takes place over a period of three to four weeks. Laboratories meet once each week and are typically three hours in length. Students are encouraged to work off hours with the understanding that they tell the instructor prior to beginning any experiments.

Week 1 comprises the "planning phase" of the project. Before the first laboratory session, students are allowed to team up with a partner, laboratory expectations are discussed (in terms of both individual and group efforts), and the teams are given time during lecture to discuss the project and outline their "plan of attack." The instructor serves as a facilitator by giving suggestions to the questions students ask without giving them a specific plan for accomplishing their goals. For example, students typically realize that a literature search might prove useful. Students who wish to "jump-in" without consulting the literature are more than welcome to do so. For those wishing to consult the literature, an online search (e.g., Chemical Abstracts Service) produces several useful references [2, 3, 6]. Although several references do give fairly detailed experimental methods, the students still must adapt the reported methods to the current problem. By week 2 these teams are usually preparing standard solutions and learning the electrochemical techniques they will use. Students who chose not to do a literature search usually realize by week 2 that they should have and quickly remedy the situation.

Unlike traditional laboratories, students are not given much background on the instruments they will use. Often, the electrochemical techniques have yet to be covered in lecture and this is their first introduction to them. Students are pointed to the appropriate sections in their texts, and several references are available for checkout from the instructor. They are also handed the instrument manufacturer's instruction manual. Before students are allowed to run samples, they are "checked out" on the instrument by the instructor and appropriate safety precautions are discussed. Once students have displayed a certain competency with an instrument they are left to their own devices. The division of work within the team is left for that team to decide and appropriate means are used to ensure that all team members take an active role (see below under Assessment).

The remaining laboratory periods are typically devoted to planning, learning the instruments, and experimentation. Voltammograms are obtained for the individual bases and for solutions containing multiple bases. Based on the ability to distinguish DNA bases in the voltammograms, teams then make a recommendation to the instructor for the optimal method and conditions. During the final laboratory period, students are required to hand in an experimental protocol that another group will use to validate their method of choice in a subsequent laboratory period.

Assessing Student Performance. Students are assessed based on both team and individual performance. Team evaluations include weekly oral and written progress reports. Oral progress reports take the form of a discussion between the instructor and one (or more) of the students in a group during class time. These generally last 1 to 2 min and a different student is asked to report each time. Written progress reports are generally collected on a biweekly basis and are limited to one half-page maximum. Students are encouraged to be brief and reports that are longer than one-half page (single-spaced; minimum 10-point font) are handed back for rewrite. These reports outline what the students have accomplished since the last report and what they intend to accomplish in the next week. Finally, teams are required to hand in an "operating" protocol for their technique of choice. The format is as individual as the teams but should have enough experimental detail so another group can duplicate their efforts. The validation of their protocols is done during a subsequent laboratory period (~1 week in length). Grades for the validation protocol laboratory are given to both the team performing the analysis and the team that generated the protocol; therefore, cooperation among the teams is important and groups realize this very quickly.

Individual evaluations include an oral quiz midway through the project to assess whether students are active members of the group, understand the chemistry, and the methods and instrumentation they are using and developing. The quiz normally lasts 10 min, and students meet with the instructor individually to answer questions. Each student also writes a final paper summarizing their findings and making comparisons among the different voltammetric techniques and the conditions under which their experiments were run. A copy

of the final-paper format is included as supplementary material.

Conclusions

The undergraduate laboratory exercise described above involves the characterization of oligonucleotide bases in DNA using voltammetry. Cyclic voltammetry is the most widely used electrochemical technique and is being used in fundamental and applied research throughout chemistry, biochemistry, and biotechnology. This experiment gives students experience in both electrochemistry and method development. It also gives students an understanding of how electrochemical techniques are being used to study and detect biologically relevant analytes.

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Supporting Materials. Three supporting files are contained in a single Zip file (http://dx.doi.org/10.1007/ s0089702595b): a laboratory handout for students, an oral quiz, and progress report guidelines.

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