# New Strategies for Electrochemical Nucleic Acid Detection

by Natasha D. Popovich and H. Holden Thorp

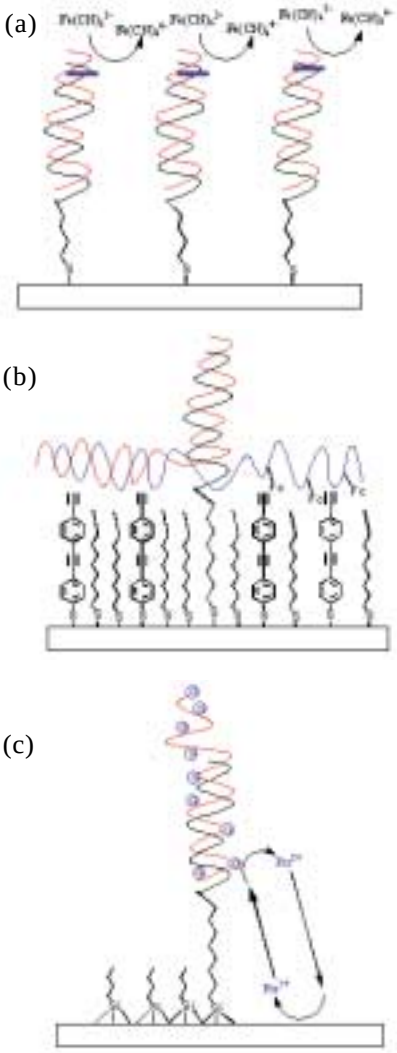


*FIG. 1. (a) Approach of Barton et al., as described in Ref. 28. Alkanethiol-modified probes (black) are attached to gold electrodes and hybridized to target (red). In the presence of redox-active intercalators (blue), reduction of ferricyanide in solution is observed; (b) Approach of Kayyem et al., as described in Ref. 29. Alkanethiol-modified capture probes (black) are attached to gold surfaces in the presence of phenylacetylene thiols. Hybridization of target (red) and signaling probes (blue) position ferrocene labels adjacent to the phenylacetylene thiols, which communicate with the gold electrode. Diluent thiols are terminated with polyethylene glycol (not shown); (c) Approach of Thorp et al. Silane-modified capture probes (black) are attached to ITO electrodes. Hybridization of target (red) allows for electrocatalytic oxidation of guanine (blue) by $Ru(bpy)_3^{3+/2+}$.*

Electrochemical technologies have received increasing attention for development of biological assays.[1,2] Electrochemical methods have the potential to allow for very low limits of detection if all of the analyte can be delivered efficiently to the electrode surface.[3] In addition, the electroactivity of the nucleobases and sugars may provide a means by which the attachment of fluorescent and radioactive labels to target nucleic acids can be avoided.[4] The ability to develop multiplexed electrode arrays could lead to dense arrays of electrically addressable microlocations for different sequences, eliminating the need for optical microscopy in obtaining spatial discrimination.[5] Finally, electrical detection has the potential to be miniaturized beyond present limits imposed by optical methods, ultimately increasing the number of DNA sequences that can be simultaneously interrogated.[6]

The electrochemical detection of nucleic acids was pioneered by Palecek and coworkers who showed that the nucleobases were electroactive when adsorbed to mercury or carbon electrodes.[7] Kuhr *et al.* later demonstrated that DNA sugars could be oxidized at copper electrodes, which provides a very sensitive method for detecting nucleic acids in flowing systems using sinusoidal voltammetry.[8] These methods show significant promise in the development of microfluidic devices with in-line electrochemical detection.[9]

The idea of an electrochemical sensor electrode that detects DNA hybridization has the additional appeal of allowing for electrodes that are functionalized with specific DNA probes and can therefore be customized for a particular sequence.[10,11] In such a sensor electrode, functionalization of the surface with the DNA probe must be done in such a way that allows for hybridization of the target from solution while also allowing for electrochemical interrogation of the surface. In this way, the DNA hybridization on the electrode would provide for separation of the desired target from a complex mixture. The separation of the target strand and detection of the target strand could then be performed with the same surface. Early efforts in this regard centered on redox-active indicators that bound more tightly to duplex DNA than to single-stranded probes.[12] Thus, when the hybridized surface was placed in contact with a solution of the indicator, the duplex DNA would concentrate the indicator at the surface, producing an enhanced signal. Mikkelsen and coworkers[13-15] first pursued such a strategy with $Co(phen)_3^{2+}$ (phen = 1,10-phenanthroline), which binds preferentially to DNA duplexes.[16] Later efforts along these lines involved $Ru(NH_3)_6^{2+}$,[17] organic dyes,[18] and intercalators functionalized with ferrocenes.[19]

While the DNA sensors provide an appealing combination of detection and strand separation, additional advances in the limits of detection have been sought through developing methods whereby large numbers of electrons can be transferred for each hybridization event that occurs. Such a strategy has been suggested by Heller *et al.* where redox enzymes can become activated by DNA hybridization.[20-23] In this article, we will discuss some new strategies involving non-enzymatic methods where multiple redox equivalents become available at the electrode for each DNA hybridization event that occurs. Representations of these strategies are shown in Fig. 1.

## Intercalator-Mediated Reduction of Ferricyanide

Hybridization-based assays for single-base mismatch detection are typically limited in sensitivity by the difference in melting temperature between the perfect duplex and the one with a single defect.[24] Barton and co-workers[25-28] have developed a new approach for detection of single-base mismatches based on charge transport through DNA films that does not rely on differential hybridization of the target nucleic acid (NA). Instead, the NA of interest is hybridized to a thiol-functionalized probe in solu-

tion and subsequently immobilized the duplexes onto gold electrodes using the self-assembled monolayer approach.[25] If the duplexes are perfectly matched, redox active intercalators such as methylene blue ($MB^+$) give a well-defined electrochemical signal upon intercalation into the film. $MB^+$ binding is primarily constrained to the top of the densely packed monolayer, requiring charge transport through the DNA films. If there is a mispaired base in the duplex between the intercalator and the electrode, the signal from the intercalator cannot be detected because charge-transfer through DNA is interrupted by the mismatch. Representative data obtained using chronocoulometry as the interrogation method are shown in Fig. 2, which illustrates discrimination between the fully complementary sequence and targets with a single-base mismatch. The main advantage of this detection method is that it is not a measure of differential hybridization and therefore can detect all single-base mismatches, including thermodynamically stable GT and GA mismatches, without stringent hybridization conditions.

The sensitivity and selectivity of this single-base mismatch detection method can be improved by using an electrocatalytic cycle involving the intercalator and freely diffusing ferricyanide, which is electrostatically repelled from the DNA.[25,28] This approach is illustrated in Fig. 1a. The intercalator, $MB^+$, is reduced by the electrode, and the reduced form of the intercalator, leukomethylene blue, reduces ferricyanide in solution. This cycle regenerates the oxidized form of the intercalator, which can then participate in more electron-transfer events. In this manner, the surface-bound DNA is repeatedly interrogated. Longer interrogation times result in greater absolute signals, as well as increased discrimination between fully complementary and mismatched DNA.

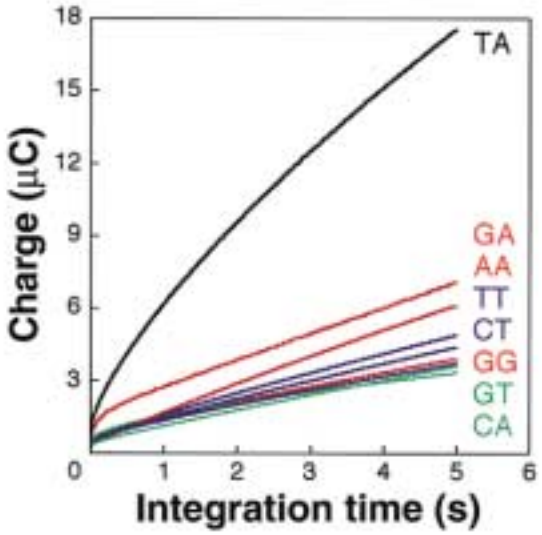


*FIG. 2. Results of chronocoulometry studies on films such as that shown in Fig. 1a with individual curves corresponding to the native duplex (TA) and duplexes containing the other mismatches in the same position. Reproduced by permission of the Nature Publishing Group (see Ref. 28).*

This detection method was implemented on a chip platform using microelectrodes and small quantities of sample. The total charge accumulated at the electrode is linearly proportional to the electrode area at electrodes ranging in diameter from 30 to 500 μm. On a 30-μm electrode, it was possible to detect approximately $10^8$ molecules. The signal from single-stranded DNA was similar to films with no mismatch. This observation can be explained by the fact that the gold surface is more exposed in the case of the single-stranded monolayer, thus allowing the $MB^+$ and ferricyanide direct access to the electrode.

## Sandwich Assays Involving AC Voltammetry

Kayyem *et al.* described an approach for electrochemical detection of DNA in a hybridization-based assay based on the use of a ferrocene-labeled signaling probe that hybridizes to the target NA near the site of probe binding.[29-31] Capture probes are immobilized onto gold electrodes via thiol-derivatized phenylacetylene bridges that allow for strong electronic coupling between the ferrocene labels and the gold electrode.[30] Site-specific ferrocene-modified DNA oligonucleotides are synthesized using phosphoramidite chemistry and exhibit the same hybridization efficiency as the unmodified probes.[29] When hybridization of the target NA to the capture and signaling probe occurs, ferrocene is oxidized and signal measured through the phenylacetylene bridge. This method is represented in Fig. 1b. The signaling probe that is not hybridized to the target does not contribute to the signal because the remainder of the monolayer co-deposited with the DNA probes is made of alkane bridges that do not provide high electronic coupling to the electrode. Thus, signaling probes and other redox-active cell debris non-specifically bound to the electrode

do not produce detectable currents. The sequence for the capture and signaling probes must be chosen carefully, because the ferrocene label must be close to the phenylacetylene bridge to observe electron transfer.[30] This feature allows this method to be used in complex sample matrices, such as whole blood, without removing other electroactive materials.

In the method of Kayyem *et al.*, AC voltammetry is used for interrogation of the electrode since this method allows for repeated collection of the electrons from the ferrocene labels.[32,33] Hence, this detection method is not truly electrocatalytic, but does have a built-in signal amplification strategy that results from the interrogation method (Fig. 3). This approach has been developed into an instrument that can simultaneously detect 48 different sequences using inexpensive gold electrode arrays on printed circuit boards.[29]

To achieve rapid and accurate detection of single-base mismatches using the ferrocene-phenylacetylene system, two different metal-containing signaling probes with different redox potentials were developed.[31] The two ferrocenyl oligonucleotide derivatives can be readily distinguished electrochemically based on the difference in their redox potentials. The use of two signaling probes allows for clear discrimination between the wild-type and mutant target sequences. To detect all single-base mismatches, it would be necessary to synthesize four different redox labels and incorporate them into signaling probes.




*FIG. 3. AC voltammograms of electrodes prepared with capture probes for HIV as shown in Fig. 1b after exposure to the target HIV sequence (solid line) or a control sequence corresponding to hepatitis C virus. Reproduced by permission of the American Society for Investigative Pathology and the Association for Molecular Pathology (see Ref. 29).*

## Electrocatalytic Oxidation of Guanine

We have developed a method for the detection of nucleic acids based on the electrochemical oxidation of guanine residues with the mediator $Ru(bpy)_3^{2+}$ (bpy = 2,2'-bipyridine).[10,34] The $Ru(bpy)_3^{2+}$-guanine electron transfer is observed as a current enhancement in the oxidation wave of $Ru(bpy)_3^{2+}$ through an EC' mechanism:[35-37]

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \quad (1)$$

$$Ru(bpy)_3^{3+} + \text{guanine} \rightarrow Ru(bpy)_3^{2+} + \text{guanine}^+ \quad (2)$$

The regenerated reduced mediator is again oxidized at the electrode, completing a catalytic cycle. The current produced during each oxidation of $Ru(bpy)_3^{2+}$ is measured and reflects the amount of guanine present in the sample. In contrast to direct oxidation of guanine at an electrode, the electron transfer reaction between $Ru(bpy)_3^{3+}$ and guanine is very fast ($k \sim 10^6$ $M^{-1}$ $s^{-1}$).[36] The rapid electron transfer between $Ru(bpy)_3^{3+}$ and guanine has been primarily attributed to the nearly identical standard redox potentials of guanine and $Ru(bpy)_3^{2+}$ and the low reorganization energy of the Ru(III/II) couple.[38] The high sensitivity of this method is based on the electrocatalytic nature of the reaction between $Ru(bpy)_3^{3+}$ and guanine, as well as the observation that multiple electrons are obtained per each guanine residue.[10] In addition, the target NA contains multiple guanine residues, which can all be oxidized at the electrode surface.

Armistead and Thorp used mediated oxidation of guanine residues to detect PCR products directly immobilized onto indium tin-oxide (ITO) electrode surface via the interaction between phosphate groups and the oxide surface.[39,40] This NA immobilization method results in sub-monolayer coverages of single-stranded NA. Adsorbed guanines did not show significant direct oxidation current but did act as a substrate for the electrocatalytic oxidation by $Ru(bpy)_3^{2+}$. The electrocatalytic current was a linear function of the amount of the immobilized NA with a slope of 0.5 μA/pmol guanine. In this study, electrodes modified with a 1497-bp PCR product from the HER-2 gene produced detectable catalytic currents with as little as 550 amol adsorbed, giving a detection limit of 44 amol/mm$^2$.

The ITO used for mediated guanine oxidation exhibits very little water oxidation current at the high potentials needed to achieve electrocatalysis.[41,42] The microstructure of ITO films has a profound effect on the electron-transfer properties of the films, and their use as electrodes. Most efficient electron transfer between $Ru(bpy)_3^{2+}$ and ITO is achieved at polycrystalline ITO films, presumably because of the higher density of defect sites along the grain boundaries or defect sites caused by substitutional Sn in the polycrystalline vs. amorphous films. These electrodes can then be modified with probes via phosphonate self-assembled monolayers[43] and silane overlayers.[44] The surface chemistry and roughness of ITO films have a profound effect on

the solid phase immobilization. ITO films used in this NA detection method have a grain-subgrain structure typical of dc-magnetron sputtered films and an average rms surface roughness of 2-4 nm (Fig. 4).[42]

Silane-modified probes can be attached to electrodes such as that shown in Fig. 4. These electrodes are then exposed to complex mixtures containing targets that correspond to the immobilized probes, producing hybridization on the surface. The silane-modified electrodes are washed to remove unhybridized biomolecules and then exposed to $Ru(bpy)_3^{2+}$. Electrochemical interrogation can then be performed by cyclic voltammetry, chronoamperometry, or chronopotentiometry. Representative chronopotentiometry data obtained for detection of ApoA1 mRNA target composed of 973 nucleobases (which includes 305 guanines) at a 200 µm ITO electrode show a detection limit of this method in a microtiter plate format of <1 fmol of target in a 50-µL sample (Fig. 5).

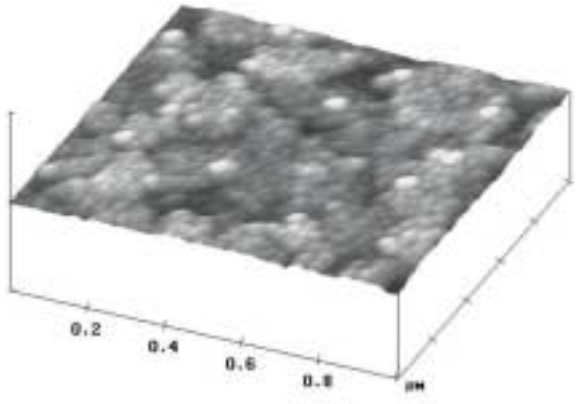


*FIG. 4. AFM image of ITO films used to prepare electrodes shown in Fig. 1c.*

Electron-transfer rates for oxidation of guanine with polypyridyl transition metal complexes vary depending on whether guanine is in single-stranded or double stranded DNA, primarily because of the difference in solvent accessibility of guanine.[35] This effect is only observed at high salt concentrations, where little pre-concentration of metal complex on the DNA occurs.[45] The base paired to guanine also has an effect on electron-transfer kinetics, with mismatches giving rates that are between those for single-stranded DNA and a perfect match. In fact, all the possible mismatches give rate constants that can be distinguished using cyclic voltammetry. However, in order to detect single-base mismatches reliably, the site of interest needs to be interrogated separately from guanines present in the sequence. This can be accomplished using a guanine derivative, such as 8-oxoguanine, which is selectively oxidized with $Os(bpy)_3^{2+}$ at a potential of 0.85 V vs Ag/AgCl.[46] The catalytic current enhancement for the 8-oxoguanine reaction with $Os(bpy)_3^{2+}$ increases in the order of 8-oxoG-C ~ 8-oxoG-T < 8oxoG-G < 8-oxoG-A < 8-oxoG, the same order observed for guanine/Ru(III). Ropp and Thorp[46] used this site-selective mismatch sensitivity to detect a TTT deletion in synthetic oligonucleotides that is important in cystic fibrosis. The authors used a probe oligonucleotide that contained 8-oxoG opposite the TTT in the cystic fibrosis gene. When hybridized to a mutant gene that does not contain the TTT sequence, 8-oxoguanine is in a single-stranded A8-oxoGA bulge and gives significantly higher catalytic current enhancement when compared to the signal obtained from the 8-oxoG hybridized to the wild-type gene. In fact, the rate constant for oxidation of 8-oxoG in the bulge has been found to be very close to the rate constant of the 8-oxoG in the single-stranded form of the probe. This change in signal is more dramatic than the readily detectable changes in the catalytic signal resulting from single-base mismatches.

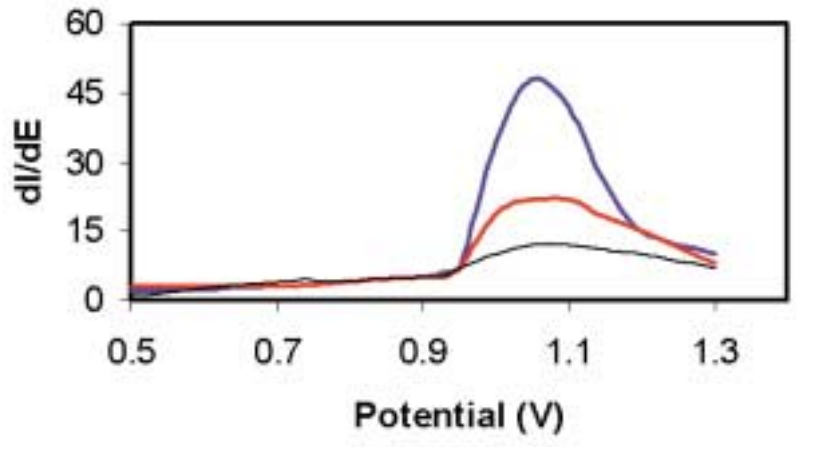


*FIG. 5. Chronopotentiometry of 200 µm ITO electrodes prepared as in Fig. 1c after exposure to 1 fmol (red) and 10 fmol (blue) of ApoA1 mRNA in the presence of $Ru(bpy)_3^{2+}$. Black curve shows the response of $Ru(bpy)_3^{2+}$ after exposure of the electrode to a nonsense target.*

## Prospects for the Future

In designing genomic assays and tests, electrochemical sensors for nucleic acids offer the prospects of lower limits of detection, greater miniaturization, and the elimination of optics. These advantages parallel those evident in the transition to electrochemical methods for glucose sensing. Considerable effort has now been directed toward many of the requisite steps needed to realize these advances. Numerous strategies are now available for attaching DNA to electrodes via connections that are robust but that also allow electron transfer to the underlying electrode. Approaches such as those described here are available for increasing the signal from hybridized targets on sensor electrodes. In addition, the application of potentials to solid surfaces can be used to increase the local concentration of nucleic acids at functionalized electrodes,[47] which could ultimately lead to increased hybridization efficiencies at probe-modified electrodes. These concepts, combined with related advances in sample preparation and molecular biology, will ultimately lead to compact sensors for economical utilization of genomic information. ■

## Acknowledgments

We thank our many talented co-workers at Xanthon and UNC-Chapel Hill who have contributed to these studies. We thank S. S. Wong for help with the cover artwork.

## References

1. W. G. Kuhr, *Nature Biotech.*, **18**, 1042 (2000).
2. E. Palecek, *Talanta*, **56**, 809 (2002).
3. R. M. Wightman, *Anal. Chem.*, **53**, 1125A (1981).
4. E. Palecek, in *Topics in Bioelectrochemistry and Bioenergetics*, G. Millazzo, Ed., Vol. 5, pp. 65-155, Wiley, New York (1983).
5. S. J. Park, T. A. Taton, and C. A. Mirkin, *Science*, **295,** 1503 (2002).
6. F.-R. F. Fan, J. Kwak, and A. J. Bard, *J. Am. Chem. Soc.*, **118**, 9669 (1996).
7. E. Palecek, *Electroanalysis*, **8**, 7 (1996).
8. P. Singhal and W. G. Kuhr, *Anal. Chem.*, **69**, 4828 (1997).
9. S. A. Brazill, P. H. Kim, and W. G. Kuhr, *Anal. Chem.*, **73**, 4882 (2001).
10. H. H. Thorp, *Trends Biotechnol.*, **16**, 117 (1998).
11. J. F. Kayyem, *Clin. Chem.*, **45**, S12 (1999).
12. S. R. Mikkelsen, *Electroanalysis*, **8,** 15 (1996).
13. K. M. Millan and S. R. Mikkelsen, *Anal. Chem.*, **65**, 2317 (1993).
14. K. M. Millan, A. Saraullo, and S. R. Mikkelsen, *Anal. Chem.*, **66**, 2943 (1994).
15. J. Wang, X. Cai, G. Rivas, H. Shiraishi, P. A. M. Farias, and N. Dontha, *Anal. Chem.*, **68**, 2629 (1996).
16. M. J. Carter and A. J. Bard, *J. Am. Chem. Soc.*, **109**, 7528 (1987).
17. A. B. Steel, T. M. Herne, and M. J. Tarlov, *Anal. Chem.*, **70**, 4670 (1998).
18. K. Hashimoto, K. Ito, and Y. Ishimori, *Anal. Chem.*, **66**, 3830 (1994).
19. T. Ihara, M. Nakayama, M. Murata, K. Nakano, and M. Maeda, *Chem. Commun.*, 1609 (1997).
20. C. N. Campbell, D. Gal, N. Cristler, C. Banditrat, and A. Heller, *Anal. Chem.*, **74**, 158 (2002).
21. T. deLumley-Woodyear, C. N. Campbell, and A. Heller, *J. Am. Chem. Soc.*, **118**, 5504 (1996).
22. T. deLumley-Woodyear, C. N. Campbell, E. Freeman, A. Freeman, G. Georgiou, and A. Heller, *Anal. Chem.*, **71**, 535 (1999).
23. F. Patolsky, A. Lichtenstein, and I. Willner, *Nature Biotech.*, **19**, 253 (2001).
24. M. Chee, R. Yang, E. Hubbell, A. Berno, X. C. Huang, D. Stern, J. Winkler, D. J. Lockhart, M. S. Morris, and S. P. A. Fodor, *Science*, **274**, 610 (1996).
25. S. O. Kelley, E. M. Boon, J. K. Barton, N. M. Jackson, and M. G. Hill, *Nucl. Acids Res.*, **27**, 4830-4837 (1999).
26. S. O. Kelley, J. K. Barton, N. M. Jackson, and M. G. Hill, *Bioconjugate Chem.*, **8**, 31 (1997).
27. S. O. Kelley, N. M. Jackson, M. G. Hill, and J. K. Barton, *Angew. Chem. Int. Ed. Engl.*, **38**, 941 (1999).
28. E. M. Boon, D. M. Ceres, T. G. Drummond, M. G. Hill, and J. K. Barton, *Nature Biotech.*, **18**, 1096 (2000).
29. R. M. Umek, S. W. Lin, J. Vielmetter, R. H. Terbrueggen, B. Irvine, C. J. Yu, J. F. Kayyem, H. Yowanto, G. F. Blackburn, D. H. Farkas, and Y. P. Chen, *J. Molec. Diagn.*, **3**, 74 (2001).
30. S. Creager, C. J. Yu, C. Bamdad, S. O'Connor, T. MacLean, E. Lam, Y. Chong, G. T. Olsen, J. Luo, M. Gozin, and J. F. Kayyem, *J. Am. Chem. Soc.*, **121**, 1059 (1999).
31. C. J. Yu, Y. J. Wan, H. Yowanto, J. Li, C. L. Tao, M. D. James, C. L. Tan, G. F. Blackburn, and T. J. Meade, *J. Am. Chem. Soc.*, **123**, 11155 (2001).
32. S. E. Creager, and T. T. Wooster, *Anal. Chem.*, **70**, 4257 (1998).
33. S. D. O'Connor, G. T. Olsen, and S. E. Creager, *J. Electroanal. Chem.*, **466**, 197 (1999).
34. D. H. Johnston, T. W. Welch, and H. H. Thorp, *Metal Ions Biol. Syst.*, **33**, 297 (1996).
35. D. H. Johnston, K. C. Glasgow, and H. H. Thorp, *J. Am. Chem. Soc.*, **117**, 8933 (1995).
36. D. H. Johnston and H. H. Thorp, *J. Phys. Chem.*, **100**, 13837 (1996).
37. L. P. Zhou, and J. F. Rusling, *Anal. Chem.*, **73**, 4780 (2001).
38. V. A. Szalai and H. H. Thorp, *J. Phys. Chem. B*, **104**, 6851 (2000).
39. P. M. Armistead and H. H. Thorp, *Anal. Chem.*, **72**, 3764 (2000).
40. P. M. Armistead and H. H. Thorp, *Anal. Chem.*, **73**, 558 (2001).
41. H. Y. Yeom, N. Popovich, E. Chason, and D. C. Paine, *Thin Solid Films*, **411**, 17 (2002).
42. N. D. Popovich, S. S. Wong, B. K. H. Yen, and D. C. Paine, *Anal. Chem.*, **74**, 3127 (2002).
43. N. D. Popovich, A. E. Eckhardt, J. C. Mikulecky, M. E. Napier, and R. S. Thomas, *Talanta*, **56**, 821 (2002).
44. A. Eckhardt, E. Espenhahn, M. Napier, N. Popovich, H. Thorp, and R. Witwer, in *DNA Arrays: Technologies and Experimental Strategies*, E. V. Grigorenko, Ed., pp. 39-60, CRC Press, Boca Raton, FL (2001).
45. M. F. Sistare, R. C. Holmberg, and H. H. Thorp, *J. Phys. Chem. B*, **103**, 10718 (1999).
46. P. A. Ropp and H. H. Thorp, *Chem. Biol.*, **6**, 599 (1999).
47. C. Edman, D. Raymond, D. Wu, E. Tu, R. Sosnowski, W. Butler, M. Nerenberg, and M. Heller, *Nucleic Acids Res.*, **25**, 4907 (1998).

### About the Authors

***Natasha Popovich*** *obtained her PhD with Dennis C. Johnson at Iowa State University working on electrocatalytic oxidation of amino acids at oxide electrodes. After that, she was Electrochemistry Manager at Xanthon, Inc. in Research Triangle Park, North Carolina. Dr. Popovich is now Senior Scientist at Home Diagnostics, Inc., in Fort Lauderdale, Florida.*

***Holden Thorp*** *is Professor of Chemistry at the University of North Carolina at Chapel Hill where he also serves as the Director of the Morehead Planetarium and Science Center.*