

Final Report Executive Summary

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Title: Synthesis and Characterization of an Electrochemical Peptide Nucleic Acid Probe

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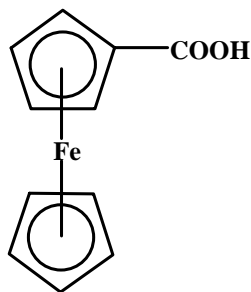
Research Category: Small Grants for Exploratory Research (SGER)

Project Period: July 1, 2000 – December 31, 2004

Objective(s) of the Research Project: The objective of this research project was to develop a non-radioactive nucleic acid probe for the detection of target sequences that has good stability to storage and that used a simple, portable assay format. An electrochemical method of detection was chosen because of the sensitivity and ease of use for detection of the hybridized probe. Electrochemical methods are fast, simple and relatively inexpensive. After the hybridization step, simply separate the hybridized probe from the unhybridized probe, place a set of electrodes into the solution, and measure the current. The instrumentation is simple to use and portable, similar to a typical pH meter.

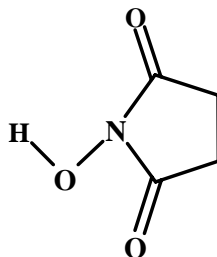
Summary of Findings:

I. Synthesis and Characterization of Ferrocenyl Peptide Nucleic Acid. An electroactive molecule, ferrocene, was covalently attached to a peptide nucleic acid strand (PNA; base sequence 3'-GCT-TTT-GTG-TAC) using the method of Takenaka et al with some modifications. Briefly, 0.22mmol of ferrocenemonocarboxylic acid and 0.25 mmol of N-hydroxysuccinimide were solubilized in 2 mL of dioxane. In a separate reaction vial, 0.25 mmol of dicyclohexylcarbodiimide was solubilized in 0.5 mL of dioxane. Next, the ferrocenemonocarboxylic acid and N-hydroxysuccinimide solution was slowly added with mixing to the reaction vial containing the dicyclohexylcarbodiimide solution and the mixture was incubated in the dark for 2 hr. The mixture was filtered through Celite using chloroform and the ferrocenylsuccinimide ester was rotary evaporated to dryness. Then, 2 mL of the ferrocenylsuccinimide ester was eluted through a silica gel column using chloroform, rotary evaporated to dryness and was dissolved in 1mL dimethylsulfoxide. Both the ^{13}C and ^1H NMR of the intermediate confirmed that we synthesized the N-hydroxysuccinimide ester of ferrocene.

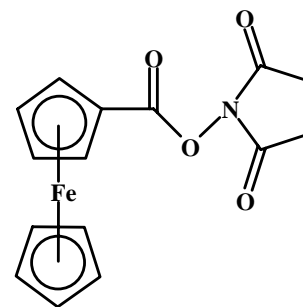
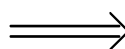


Ferrocenemonocarboxylic Acid

+

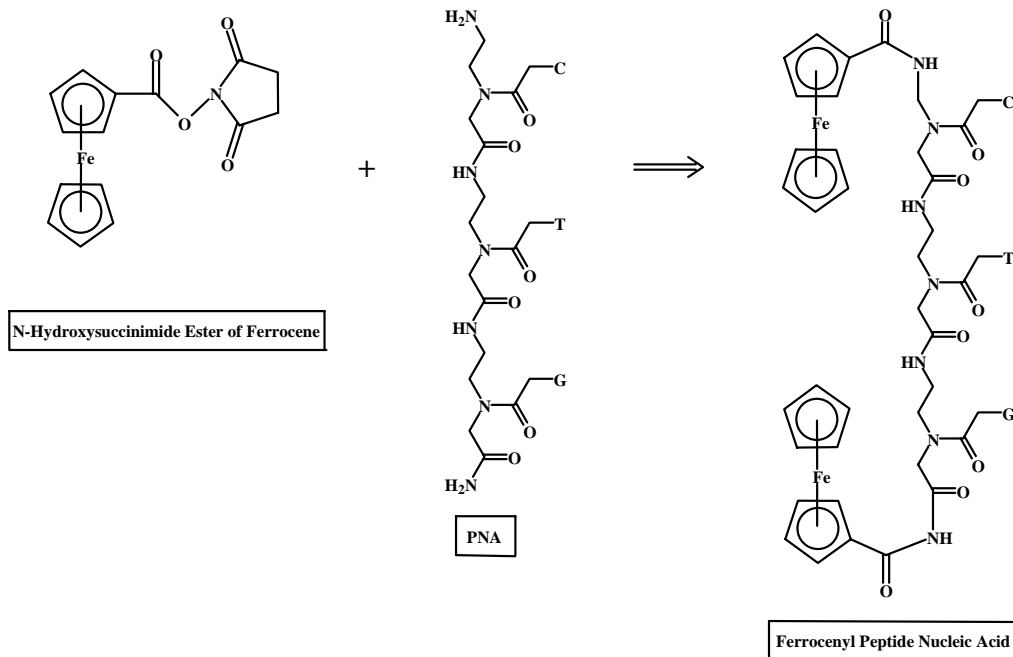


N-Hydroxysuccinimide



N-Hydroxysuccinimide Ester of Ferrocene

An 800 μL aliquot of the ferrocenylsuccinimide ester was mixed in another reaction vial with 200 μL aliquot of the peptide nucleic acid solution (PNA; 79 nmol) and stirred in the dark at room temperature for 2 hr.

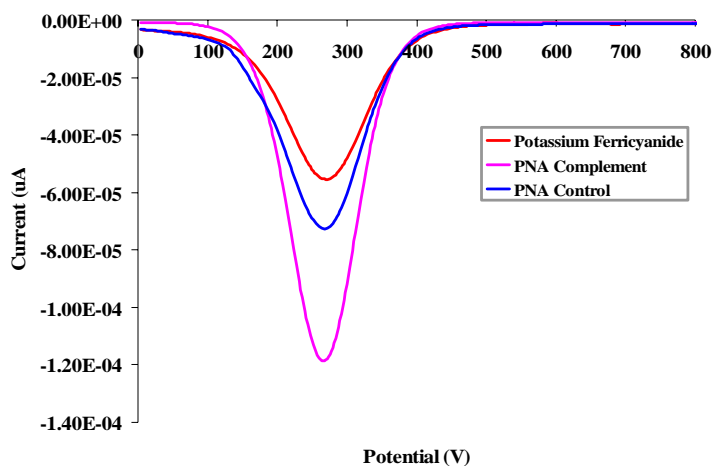


The ferrocenyl PNA was separated from the reaction mixture using gel filtration (Sephadex G-15; 0.10 M sodium phosphate buffer, pH 7). A colorimetric BCA (bicinchoninic acid) assay was performed on all the fractions to test for the presence of peptide bonds. The absorbencies at 260 nm (absorbance maximum for the nitrogen bases) and 450 nm (absorbance maximum for ferrocene) were also determined for each of the fractions. The fractions that tested positive using the BCA assay were pooled and characterized using cyclic voltammetry and UV-visible spectroscopy. Absorption at a wavelength of 450 nm (λ_{max} for ferrocene) and voltammetry analysis of the ferrocenyl PNA sample was used to confirm the incorporation of the ferrocene into the PNA strand. The scan of the ferrocenyl PNA from 250-500nm had peaks at 260 nm and 450nm confirming the incorporation of ferrocene into the PNA molecule.

II. Synthesis and Immobilization of Azido-Tailed Target Oligoribonucleotide. Another aspect of the project was the synthesis and immobilization of azido-tailed target oligoribonucleotide. A terminal azido modification was made to the target oligoribonucleotide (base sequence 5'-rCGAAAACACAUCAAAAAAA) and the control oligoribonucleotide (base sequence 5'-rCGAAUUGUCAUCAAAAAAA) using 8-azido ATP and poly (A) polymerase following manufacture's protocol. Briefly, the reaction mixture consisted of 40 mM Tris-HCl (pH 7), 10 mM MgCl₂, 2.5 mM MnCl₂, 25 mM NaCl, 55 μM 8-azidoATP, 25 μg of the oligoribonucleotide, 50 μg of BSA and 10 Units of poly (A) polymerase. The reaction mixture (500 μL total volume) was incubated at 37°C for 4 hr. After a 10 min incubation at 75°C to stop the enzymatic reaction, the azido modified oligoribonucleotide was purified by gel filtration chromatography (Sephadex G-15; 0.10 M sodium phosphate buffer, pH 7). The absorbance at 260 nm was determined for each fraction and an absorbance scan from 230 – 340 nm was

obtained for fractions containing the oligoribonucleotide. To verify the presence of the azido group incorporation into the oligoribonucleotide, an aliquot of the sample was irradiated with short wavelength uv light ($\lambda = 254$ nm) for 2 min and rescanned under the same conditions. Next, a 100 μ L aliquot of the azido modified oligoribonucleotide was placed onto the bottom of a polystyrene containers and allowed to evaporate overnight at room temperature. The samples were irradiated for 30 s using short wavelength uv light ($\lambda = 254$ nm) to immobilize the azido modified oligoribonucleotide to the bottom of the polystyrene containers. The containers were rinsed twice using 1 mL aliquots of 0.10 M sodium phosphate buffer (pH 7). The washes were pooled and the absorbance of each was determined at a wavelength of 260 nm for computation of percent immobilization.

III. Hybridization and Electrochemical Analysis. The ferrocenyl PNA (3'-GCT-TTT-GTG-TAC) solution was heated to 65°C for 5 min and 200 μ L of the solution was placed into each polystyrene container containing immobilized oligoribonucleotide (oligoribonucleotide complement sequence: 5'-CGA-AAA-CAC-AUC; 1 base mismatch and oligoribonucleotide control sequence: 5'-CGA-AUU-GUC-AUC; 5 bases mismatch). The samples were incubated at room temperature for 1 hr. The solution was removed from the containers and the containers were rinsed twice with 1.0-mL portions of 0.10 M sodium phosphate buffer, pH 7.0. Next, 7.0 mL of a potassium ferricyanide solution (10.0 mg/mL dissolved in 0.10 M sodium phosphate buffer, pH 7.0) were added to each container. The electrodes (a glassy carbon microelectrode, Ag/AgCl reference electrode and a Pt wire auxiliary electrode) were placed in the solution and the samples were analyzed using the square wave technique using an initial potential of 0 mV, final potential of 700 mV, a sensitivity of 1 μ A/V, a quiet time of 2 s and a scan rate of 50 V. A baseline scan was made using the potassium ferricyanide solution and six scans were performed for each complement and control hybridizations. The baseline current was subtracted from the average currents of the complement and control hybridizations. The control oligoribonucleotide had a 25% mismatch in the base sequence and produced an average current of 23.691 μ A. The complement oligoribonucleotide produced an average current of 64.675 μ A. The results indicated that the ferrocenyl peptide nucleic acid hybridized to the immobilized oligoribonucleotide. An overlay of the baseline, control, and complement scans are shown below. A clear distinction can be made between the complement oligoribonucleotide and the control oligoribonucleotide (25% base mismatch).



IV. Conclusion. The synthesis of ferrocenyl PNA was simple and straightforward. The control and complement oligoribonucleotide was end labeled with azido modified adenosine residues for end immobilization of the target nucleic acid strands to the solid support. The hybridization assay was simple to perform. The ferrocenyl PNA solution were added to containers with immobilized target, incubated under appropriate conditions, and the unhybridized probe was removed by rinsing the containers. The detection step was simple and easy to perform. The electrochemical solution was added to the containers, the electrode system placed in the solution, and the current measured. This is a simple and easy hybridization assay format which can be adapted for field use. The instrumentation is relatively inexpensive and portable.

V. Relevance and Practical Application. The development of a portable and simple to use hybridization assay with good sensitivity (picomole and lower detection level) is important in the monitoring of seafood safety. Food borne illnesses can occur from eating fish and shellfish contaminated with pathogenic bacteria. Current detection methods are not suitable for rapid on-site monitoring of seafood samples for pathogenic bacterial contamination. Using an electrochemical hybridization assay format can be as portable and as simple as taking a pH reading.

Publications/Presentations:

1. J. Keen, S. Jivarajan, B. Horne, and N. Campbell (**in preparation**). Synthesis and Hybridization of Ferrocenyl Peptide Nucleic Acid.
2. N. F. Campbell and Jessica N. Keen. 2004. Synthesis and Hybridization of Ferrocenyl Peptide Nucleic Acid. 227th ACS National Meeting, Analytical Division.
3. A. C. Robinson, S. Jivarajan and N. F. Campbell. 2002. Synthesis of Ferrocene Labeled Peptide Nucleic Acid Probe. 223th ACS National Meeting, Undergraduate Division.
4. S. Jivarajan, A. C. Robinson, T. R. Taylor and N. F. Campbell. 2001. Electrochemical Detection of Hybridized Ferrocenyl Polyadenylic Acid. ACS Regional Meeting, Undergraduate Division.
5. B. E. Horne, K. M. Ratliff and N. F. Campbell. 2000. Synthesis and Electrochemical Characterization of Ferrocene-Labeled Polyadenylic Acid. 220th ACS National Meeting, Undergraduate Division.

Reference:

Takenaka, S., Uto, Y., Kondo, H., Ihara, T., Takagi, M. (1994) Electrochemically active DNA probes: Detection of target DNA sequences at femtomole level by high performance liquid chromatography with electrochemical detection. *Analytical Biochemistry* 218: 436-443.

Supplemental Keywords: Peptide Nucleic Acid Probe, Electrochemical Hybridization Assay, Ribosomal RNA, Ferrocene Nucleic Acid Probe, Electrochemical Nucleic Acid Probe, Pathogens, Bacteria.

Relevant Web Sites: None

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