Synthesis, characterization and use of Ru-Fc intercalation complex as an electrochemical label for the detection of pathogen-DNA

M Díaz-Serrano¹, A Rosado¹, D Santana², E Z Vega² and A R Guadalupe¹,³

¹ Department of Chemistry, P.O. Box 23346, University of Puerto Rico, Río Piedras Campus, San Juan, Puerto Rico, 00931-3346
² Department of Biology, P.O. Box CUH 100 Rd 908, University of Puerto Rico, Humacao Campus, Humacao, Puerto Rico, 00791

Email: anlupe55@gmail.com

Abstract. This report describes the synthesis of [Ru(Fe-Phen)₂dppz]²⁺ (Ru-Fe complex) for a label-free approach to detect DNA hybridization. The Ru-Fe complex showed oxidation signals at +608 mV and +1192 mV corresponding to the RuII/III and FeII/III centers, respectively. We used the Ru-Fe complex and the Ferrocene covalently attached to the target to monitor the hybridization event of a 70-mer oligo immobilized in 10.3KD NHS-PS-NHS. The lowest target detectable concentration for the DNA fragment was around 0.4 µM.

1. Introduction

Waterborne and foodborne diseases are one of the principal public health problems worldwide. Our particular interest is on the development of nucleic acid biosensors (NAB) for the detection of pathogenic microorganisms in food and water samples. In a NAB, a ss-nucleic acid sequence is used as a probe to identify a complementary target sequence (the analyte) [1]. The detection of the hybridization process relies on the measurement of a redox-active molecule that interacts with the double strand. Two approaches to the use of redox labels can be mentioned. In one of these approaches, the redox label is conjugated to the probe or the target and the hybridization is signaled by measuring a current (or potential) or a resultant change for the case when the label is at the probe [1-4]. A second approach is the so-called label-free which is actually an indirect measurement of the hybridization process.

Ruthenium (II) polypyridyl complexes have received considerable attention because of their binding interaction with dsDNA [5-14]. [Ru(bpy)₂dppz]³⁺ and [Ru(phen)₂dppz]³⁺ (dppz = dipyrido[3,2-a: 2', 3'-c]-phenazine) have been reported as “DNA light-switching” molecules where their negligible luminescence in water is enhanced on the addition of dsDNA [6,11]. In these two complexes, the dppz ligand possesses an extended planar π-aromatic structure, which favors the complex intercalation between the base pairs of double helical DNA [5-14]. The interaction of [Ru(L₂)dppz]²⁺ complexes with dsDNA has been well studied [8,11-14] in solution. In this research, we report on the development of a NAB prototype using a polymer modified electrode together with a 70-mer probe selective to Salmonella as probes and Ruthenium-Ferrocene (Ru-Fc) bi-metallic

³ To whom any correspondence should be addressed.

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complex as a label (scheme 1). The results from these experiments were compared with prior experiments [15] where we used only ferrocene conjugated to the target to signal the hybridization event.

2. Methodology

2.1. Synthesis and characterization of the Ru-Fe complex (Scheme 1)
The dppz and Fc Phen ligands were prepared as described by Dupureur and Barton [6,9] and by Zapata and coworkers [16], respectively. The cis-Ru(Fe-Phen)$_2$Cl$_2$ was synthesized following the procedure by Z. Ji and coworkers [17]. 17.41 mg (0.067 mmol) of RuCl$_3$·xH$_2$O (x assumed to be 3) was refluxed with 70.56 mg (0.17 mmol) of Fe-Phen and 21.85 mg (0.52 mmol) of LiCl for 8 h in 10 mL of DMF under continuous stirring. The crude product was purified by suspending it in 200 mL of water:ethanol (1:1) and refluxing the mixture until all solids had dissolved (~30 min) to form a deep brown red solution. This solution was filtered and rotoevaporated to remove the ethanol in the presence of 1 g LiCl. 50 mL of acetone was added to precipitate the product. The crystals were filtered and rinsed with diethyl ether. The yield was 90%.

The resultant complex Ru(Fe-Phen)$_2$Cl$_2$ (99.61 mg, 0.11 mmol) and dppz (28.54 mg, 0.10 mmol) were refluxed overnight in 30 mL of DMF under N$_2$(g) and with continuous stirring. The volume was reduced, the solution cooled, and aqueous NH$_4$PF$_6$ added. The resulting red precipitate was filtered and rinsed with acetone followed by diethyl ether. The product was purified by size exclusion chromatography with Sephadex LH-20 and methanol:acetonitrile (1:1) as the eluent. The yield was 66%. MS -TOF, ¹H-NMR, UV-Vis and OSWV were used to characterize the ligands and the complexes. The electrochemistry was done in CH$_3$CN/0.1 M TBAP.

Scheme 1. Ru-Fc bimetallic intercalator and sandwich design of the DNA hybridization detection.

2.2. Preparation and calibration of a 70-mer DNA biosensor
Glassy carbon electrodes were cleaned and modified with a 10.3 KD NHS-PS-NHS polymer film as previously published [15]. A 20 µL of 7 µmol/L of 70-mer oligo (probe) solution was deposited at the polymer film and incubated at RT for 12 h. The probe-modified film was rinsed with 5 mL of deionized water for 5 min. The concentration and purity of the oligo solutions was previously determined by measuring the absorbance at 260 and 280 nm. A sample of pure ss-DNA with an absorbance equals to one at 260 nm contains approximately 33 mg/mL of DNA [15].

The hybridization of the immobilized probe was studied by looking at the redox process of (Scheme 1): (1) ferrocene covalently attached to the complementary 70-mer target sequence [15], and
(2) the Ru-Fe complex intercalated within the double strand. A 20 µL of the target solution was deposited at the probe-modified film, incubated at 70°C for 5 min and left at RT for 12 h. The film was rinsed successively with 5 mL of PBS (pH 6.9). An OSWV was run in PBS/0.103 M NaCl (µ=0.271 M) from 200 to 1300 mV. The sensor was exposed to a 1.58 X 10⁻⁴ M Ru-Fe solution in PBS/0.103 M NaCl for 4 hr. This solution was prepared by mixing a 50 µL aliquot of a 7.9 x 10⁻⁴ M Ru-Fe acetonitrile solution with 200 µL of PBS/0.103 M NaCl. OSWV followed under the same experimental conditions. Calibration curves were constructed varying the target concentration from 0.8 µM to 0.4 µM. The current density from the oxidation of the ferrocene in the ferrocene-labeled target and the ruthenium in the bimetallic complex were used for the construction of the calibration curves.

3. Results and Discussion

3.1. Characterization of the compounds. The electrochemical behavior of 1.3 mM Fe-Phen and 0.68 mM Ru-Fe complex was studied in CH₃CN/0.101 M TBAP with OSWV. The Ru-Fe complex showed oxidation signals at +608 mV and +1192 mV corresponding to the Ru II/III and Fe II/III centers, respectively (see Figure 1A-B). The Fe-Phen ligand showed an oxidation potential at 548 mV.

![Figure 1. Square wave voltammetry of 1.3 mM Fc-Phen (blue) and 0.68 mM [Ru(Fc-phen)₂dppz](PF₆)₂ (red) between 100 and 1400 mV in acetonitrile 0.101 M TBAP. Glassy Carbon electrode, nichrome wire and Ag/AgCl (3 M NaCl) were used as working, counter and reference electrodes respectively.](image)

3.2. Calibration of the 70-mer DNA biosensor. Figure 2A (a-c) shows a sequence of voltammograms where the 70-mer target was hybridized with its complementary 70-mer probe immobilized at the 10.3KD NHS-PS-NHS modified glassy carbon electrode. The hybridization event was followed using the ferrocene-labeled target (2A-b) and the Ru-Fe complex (2A-c). As expected, we did not observe a redox signal for the polymer neither the probe (2A-a) because they are not electroactive in the potential interval studied. The voltammogram in 2A-b shows a signal at +336 mV resulting from the ferrocene oxidation [15]. In 2A-c, the voltammogram shows anodic peaks at +1092 mV and +532 mV after exposing the modified electrode surface to the Ru-Fe complex solution. The appearance of the two oxidation peaks confirms the interaction of the Ru-Fe complex with the immobilized dsDNA.

Calibration curves for the modified electrode using both detection schemes are shown in Figure 2B. The target concentration was varied from 0.8 µM to 0.4 µM. The current density of the Fe(II) oxidation in the ferrocene label and the Ru signal in the Ru-Fe complex were reported for each single point and used to construct the calibration plot. The shape of the curve has similar features, however the raising part of the 2B-b curve is steeper compared to curve 2B-a. The lowest target detectable
concentration was around 0.4 µM for both cases. The standard deviation of each curve points varied between 0.25 µM to 0.01 µM for the ferrocene signal and 0.57 µM to 0.05 µM for Ru signal.

Figure 2. A: A 10.3 KD NHS-PS-NHS modified glassy carbon electrode with probe-target hybridization for the 70 mer. The hybridization event was followed using the ferrocene-labeled target (b) and the Ru-Fe complex (c). B: Calibration curves for a 10.3 KD NHS-PS-NHS modified glassy carbon electrode with a 70 mer oligo and its complementary target in 0.070 M PBS/ NaCl (μ = 0.271 M; pH 6.9) followed by the ferrocene-labeled target and the Ru-Fe complex. The target concentration was varied from 0.8 µM to 0.4 µM.

4. Conclusions
We synthesized and successfully characterized the ligands (Fe-phen and dppz) and the bimetallic Ru-Fe complex. The Fe-Phen showed the characteristic Fe^{II/III} process of ferrocene. Ru-Fe complex showed the expected Ru^{II/III} and Fe^{II/III} redox processes. A 70-mer DNA biosensor using a polymer modified electrode anchoring and sensing platform and the Ru-Fe complex as the electrochemical label was constructed and calibrated. OSWV confirmed the Ru-Fe complex interaction with the immobilized dsDNA. The lowest target detectable concentration was above 0.4 µM. Our results show that it is possible to use metal complexes as DNA intercalators when appropriately designed for this purpose. Current work focuses on the sensor platform and the signal to noise ratio optimization by increasing the polymer hydrophilicity and thus the diffusion of molecules within the film. This sensor could be used to study the hybridization of complementary oligo sequences and their detection in real samples.
Acknowledgements
We acknowledge the kind support of NSF-PREM (DMR-0934195), NIH-MARC (5T34GM008156-20) and NIH-RISE (2R25GM061151-09). We also thank the technical assistance of the UPR, Río Piedras Materials Characterization Center.

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