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Label-Free Electrochemical Detection of Protein Based on a Ferrocene-Bearing Cationic Polythiophene and Aptamer

Fabien Le Floch, Hoang A. Ho, and Mario Leclerc*

Department of Chemistry, Université Laval, Québec City, Qc, Canada G1K 7P4

Two label-free electrochemical methods for the detection of human α-thrombin using a water-soluble, ferrocene-functionalized polythiophene transducer and a single-stranded oligonucleotide aptamer probe are described. The first approach is a direct method in which the recorded current decreases upon addition of the targeted protein. The second one requires more steps and the additional utilization of PNA probes and nuclease enzyme. This indirect method leads to an increase of the electrical signal as a function of the concentration of human α-thrombin with a detection limit of 75 fmol.

Proteins play a key role in biochemical systems and studies of their rapid, sensitive, and specific identification remains a great challenge that attracts a lot of attention worldwide. One of the most interesting methods of detection is the utilization of electrochemical systems that combine miniaturization, rapidity, sensitivity, and low cost. In this way, a new possibility for the development of bioassays is the use of electroactive and photoactive conjugated polymers (CPs).[1−5] These materials are well utilized for the optical detection of proteins,[6−12] but CPs have also shown some utility as supporting matrixes or as mediator of the electrical modifications induced by the binding of the target. In these electroanalytical systems, proteins could be directly adsorbed,[13] linked through the biotin−avidin assembly during or after electropolymerization of the CPs,[14,15] or photografted.[16]

In parallel, aptamers,[17−21] synthetic oligonucleotides with characteristic 3-D structures, represent a new and interesting class of ligands to selectively bind a protein without using antibodies extracted from animals. Up to now, different techniques could be used for the detection of proteins using aptamer probes, including the following: fluorescence,[22−29] radiolabeling,[30] EQCM measurements,[31,32] and AFM.[33,34] Concerning electrochemical detection, Xu et al. have used electrochemical impedance spectroscopy on hairpin aptamer probes without using any labeling,[35] while Radi et al. have utilized impedimetry to quantify analyte present onto the electrode surface.[36] Ikebukuro et al. have employed aptamer labeled with glucose dehydrogenase to detect thrombin in an amperometric way.[37] Voltammetry was also used to detect elec-

* To whom correspondence should be addressed. E-mail: mario.leclerc@chm.ulaval.ca.

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troactive lysozyme collected with aptamer-grafted magnetic beads,[38] Xiao et al. used ferrocene-labeled aptamer probes as an electroactive molecular beacon,[39] whereas King et al. have employed voltammetry to detect rolling circle amplification products for real-time protein detection with circular DNA aptamers.[40] Following these studies, we report here two new, rapid, label-free electrochemical detections (direct and indirect) of proteins. These analytical techniques involve the utilization of a single-stranded (ss) DNA aptamer, a cationic electroactive polymeric transducer, and S1 nuclease (employed only for the indirect method).

EXPERIMENTAL SECTION

Materials. Thrombin aptamer oligonucleotide (X1) 5′-GGT TGG TGT GGT TGG-3′, thrombin thiolated aptamer oligonucleotide (HS-X1) 5′- HS-C_6-GGT TGG TGT GGT TGG-3′, and thiolated DNA arbitrary sequence (HS-X2) 5′- HS-C_6-CAT GAT TGA ACC ATC CAA CA-3′ were synthesized by Integrated DNA Technology (Coralville, IA). HS-PNA aptamer reverse complementary probes (HS-Y1) 5′ Cys-O-CCA-ACC-ACA-CCA-ACC 3′ was provided by Applied Biosystems (Foster City, CA). The 2-μL aliquots of thiolated probes concentrated at 400 μM were prepared under an inert atmosphere and kept frozen for several months. Frozen aliquots were diluted to 4 μM under inert atmosphere conditions just before use in sterilized water for PNA and in PB (KH_2PO_4 0.5M/K_2HPO_4 0.5M) for DNA. Human α-thrombin was obtained from Haematologic Technologies, Inc. (M_w = 36 700 g·mol^{-1}, concentration 9.9 mg·mL^{-1}). Bovine serum albumin (BSA MW = 68 000, 10.1 mg·mL^{-1}) and electrochemical salts were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). S1 nuclease was provided by Invitrogen (Invitrogen Canada Inc., St. Burlington, ON, Canada). The electrochemical transducer, a cationic polythiophene bearing a ferrocene substituent (polymer 1), was prepared as described in a previous publication.[41]

Probe Immobilization on Gold Electrodes. After an electrochemical oxidation with a cyclic voltammetry sweep from 0 to 1300 mV and polishing on felt pad (Buehler 1200-grit Microcut paper disks) with 0.3- and 0.05-μm alumina (Buehler Gamma Micropolish deagglomerated alumina, Lake Bluff, IL), the 2-mm^2 gold electrodes (BAS, BioAnalytical Systems Inc.) were vigorously rinsed three times with sterilized water. After drying with a flow of inert gas, electrodes were put face up in plastic tubes containing 200 μL of sterilized water under argon and probes were grafted by depositing a 1 μL of a 2 μM solution of HS-Y1 in PB (0.5 M K_2HPO_4/0.5 M KH_2PO_4) for direct detection of human α-thrombin or a 1-μL drop of 3 μM HS-Y1 in sterilized water for S1 nuclease-assisted detection. The tubes were sealed to prevent water evaporation. After 45 min, the electrodes were rinsed with sterilized water and 0.1 M NaCl aqueous solution to desorb nongrafted probes. Probe density was estimated to $\Gamma = 2.0 \pm 0.4 \times 10^{-11}$ mol·cm^{-2} for aptamer DNA probes used for the direct detection and $\Gamma = 2.3 \pm 0.3 \times 10^{-11}$ mol·cm^{-2} for PNA probes used in the indirect detection system. See Supporting Information for detailed experimental procedures.

Preparation of the Analytical Samples. (a) Direct Method 1. A 1-μL aliquot of different concentrations of human α-thrombin diluted in 6× SSPE (Omnipur), 0.03% poly(vinylpyrrolidone) (PVP), 30% formamide, and NaOH pH 8.5 was deposited on the grafted electrode for 30 min to allow the thrombin−aptamer binding. After the electrode was rinsed with water, the electrodes were dipped for 1 min in a solution of 100 μL (10 μM) of the cationic polythiophene, rinsed again with water, and electrochemically tested. The same operations were used for BSA and the mixed BSA−human α-thrombin solutions.

(b) Indirect Method 2. A 2-μL aliquot (40 μM, 80 pmoles) of human α-thrombin aptamer diluted in PB (0.5 M K_2HPO_4/0.5 M KH_2PO_4), 0.03% PVP, and 30% formamide was incubated for 30 min with 25-μL aliquots (sterilized H_2O, 100 mM NaCl) of several proteins (BSA, human α-thrombin, or a 50:50 mixture of both) at different concentrations. A solution of S1 nuclease was prepared from stock solution (1000 units/μL in average) by diluting with a buffer solution (20 mM Tris-HCl pH 7.5, 0.1 mM zinc acetate, 50 mM NaCl, 50% (v/v) glycerol) to obtain a concentration of 2 units·μL^{-1}. Before use, 1 μL of the previous diluted solution of S1 was activated by 5 μL of Zn^{2+} solution (300 mM sodium acetate (pH 4.6), 10 mM zinc acetate, 50% (v/v) glycerol). The final enzymatic solution was added to the aptamer−thrombin complex previously aliquoted. The enzymatic reaction was performed during 10 min at 38 °C. The enzyme activity was then stopped with 5 μL of 0.2 mM EDTA/NaOH (pH 8) in water, and the strong binding between the protein and its aptamer was broken up by dipping the aliquots in boiling water for 10 min. By depositing 1 μL of the analytical solution on the HS-Y1-modified electrode, the released aptamer was then ready to be hybridized with the aptamer complementary probe PNA strand and then be electrochemically quantified. The 1-μL drop was deposited on the electrodes and kept 30 min in humid atmosphere to prevent evaporation. As before, after rinsing the electrode with water, the electrodes were dipped for 1 min in a 10 μM aqueous solution of the cationic polythiophene, rinsed again with water, and electrochemically tested. It should be noted that too high enzyme concentrations may also destroy ss-DNA aptamers linked to the protein.

Electrochemical Measurements. A standard three-electrode system was used with the Epsilon potentiostat from BASi (BASi, BioAnalytical Systems Inc.). The system consisted of a gold working electrode, a platinum wire counter electrode, and an Ag/AgCl (saturated KCl) reference electrode. Working electrodes were freshly prepared for each analysis. Detection was obtained by depositing 1 μL of the test sample during 30 min onto the grafted electrodes. All electrochemical experiments were conducted in an electrolytic solution of water with 0.1 M $LiClO_4$ (Aldrich) at room temperature (24 °C). Square-wave voltammetry (SWV) was performed for revealing the electrode surface. The potential was scanned from 0 to 1100 mV with a step potential of 4 mV, a square-wave frequency of 15 Hz, and a square-wave amplitude of 25 mV. The electrochemical signal was provided by the ferrocene-bearing cationic polythiophene after soaking the testing electrodes 1 min in a 10 μM solution of an aqueous solution of the polymer. All peaks are reported against the oxidation potential of $FeCN_6^{4-}$ (+0.164 V vs Ag/AgCl).

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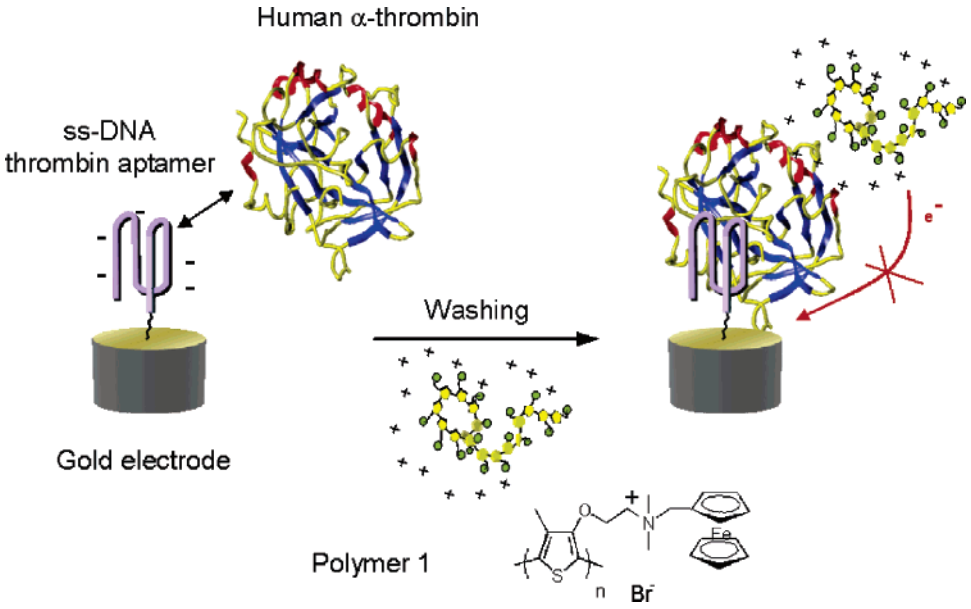

Figure 1. Chemical structure of polymer 1 and a schematic description of the direct electrochemical detection of human α-thrombin.

RESULTS AND DISCUSSION

To develop these proposed electrochemical detection methods, a cationic polythiophene bearing a ferrocene substituent (polymer 1, Figure 1)[41] was employed as the electrochemical mediator. The SWV study of polymer 1 adsorbed on gold electrodes exhibits two oxidation potentials. The first oxidation peak at 490 mV versus Ag/AgCl is well defined and attributed to the reversible oxidation of the ferrocene (Fc) group to ferrocenium (Fc^+). A second weak and broad oxidation signal ~690 mV versus Ag/AgCl is related to the reversible oxidation of the polythiophene backbone that may act as a molecular wire, thus facilitating the electron transfer of the ferrocene moieties.

Direct Detection. In these experiments, the gold electrode surface is functionalized with an appropriate oligonucleotide probe, i.e., the human α-thrombin aptamer. After probe grafting, the modified electrode is directly dipped in the analytical sample to react with human α-thrombin solutions. The electrode is carefully washed and then finally put in contact with an aqueous solution of the electroactive polymer 1. The electrochemical signal is recorded by SWV to determine whether the interaction between the aptamer and the protein occurred. In principle, without interaction between the oligonucleotide probe and the protein, a high current peak should be obtained, indicating the presence of the electroactive polythiophene electrostatically bound to the negatively charged electrode surface. In contrast, when the protein−aptamer complex is formed, no signal is expected because of the blocking capacities of the protein, which hinder the electrostatic binding of the polythiophene and therefore limit the electronic transfer to the electrode (Figure 1).

However, it is first important to take into account the experimental conditions, such as the pH, to avoid nonspecific adsorption of the analytes. Indeed, proteins are well known for their "sticking" properties. The protein adsorption on surfaces below the isoelectric point in neutral and acidic media were reported,[42,43] and we have observed that human α-thrombin was strongly (and nonspecifically) adsorbed onto the electrode in these pH conditions (see Supporting Information). For all experiments described in this section, pH 8.5 was chosen and two sequences of oligonucleotide probes were tested: X1 (specific human α-thrombin aptamer) 5′-HS-C6-GGT TGG TGT GGT TGG-3′ and X2 (nonspecific human thrombin sequence) 5′- HS-C6-CAT GAT TGA ACC ATC CAA CA-3′. In these conditions, human α-thrombin does not interact with the probe X2, which reacts freely with polymer 1 to give a strong electrochemical signal at 490 mV versus Ag/AgCl from the Fc/Fc^+ couple (Figure 2a). The same intensity, the result of free oligonucleotides, is also obtained when the analytical sample contains only a nonspecific protein such as BSA in the presence of probe X1 (Figure 2 inset). However, when the complex human α-thrombin−probe X1 is formed (even in the

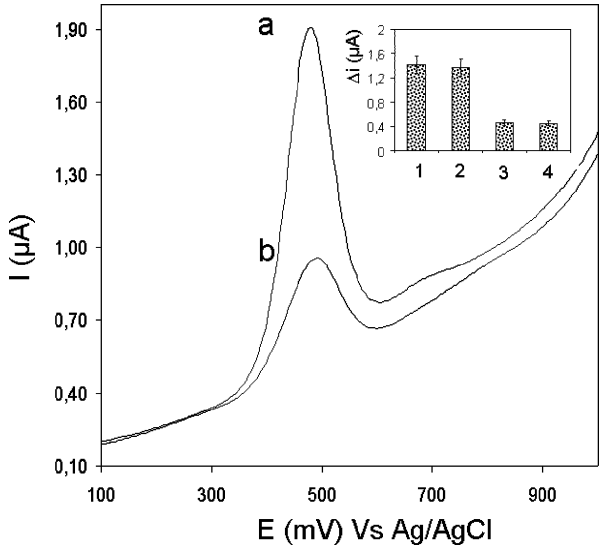

Figure 2. Direct detection of 2 μM human α-thrombin using SWV at pH 8.5 with (a) nonspecific human α-thrombin sequence (X2) and (b) specific human α-thrombin aptamer (X1). Inset: SWV signals for the X1-functionalized electrode after exposure to (1) solution without analyte, (2) BSA, (3) thrombin, and (4) mixture of BSA + thrombin (2 μM each), pH 8.5.

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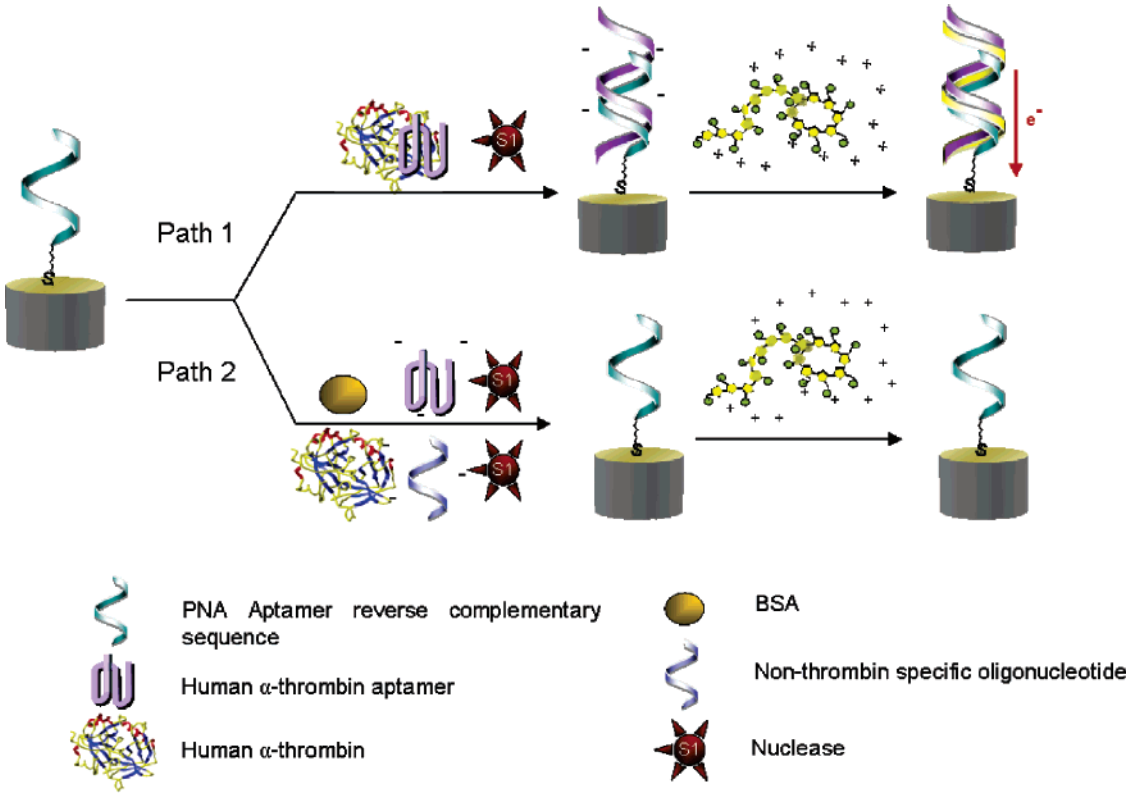

Figure 3. Indirect thrombin detection using neutral PNA probes and S1 nuclease. Path 1: A signal generated by polythiophene is detected when the sample contains human α-thrombin complex with its specific thrombin aptamer X1. Path 2: No signal occurred when BSA or nonspecific thrombin sequence was used.

presence of BSA), the electrostatic binding between the cationic conjugated polymer and ss-DNA aptamer is prevented, resulting in a lower electrochemical signal (Figure 2b and inset).

However, it should be noticed that a weak signal is always observed when the protein is detected. It seems that the entire surface could not be entirely covered with the protein that probably encounters some steric impediments. Some ss-DNA strand areas remain accessible for binding with the electroactive cationic polythiophene. Surprisingly, increasing the protein concentration has no effect on the signal intensity. In short, the direct detection of human α-thrombin requires controlled pH conditions and leads to a decrease of the electrochemical signal when the protein target is identified. Although an increasing signal is preferable for biomolecule detection, this system can be considered as an "on/off" sensor. However, no calibration curve of the protein concentration could be obtained with this approach. A significant decrease of the signal was obtained for concentrations higher than 1 μM (which means 1×10^{-12} mol of the protein). To dose the protein of interest and to avoid controlled pH conditions, we have developed another electrochemical method employing PNA probes and S1 nuclease enzyme.

Indirect Detection Using PNA Probes and S1 Nuclease. The principle of this indirect protein detection is based on the protein−aptamer complex dissociation at high temperature and on an enzymatic hydrolysis as shown in Figure 3. The specific human α-thrombin aptamer (X1) is added in excess to a solution of unknown concentration of human α-thrombin. After 30 min, during which the complex aptamer−protein can form, the S1 nuclease enzyme is added to specifically hydrolyze the free ss-DNA aptamer (Figure 3, path a). In other words, the aptamer/protein complex protects the linked ss-DNA from hydrolysis. The enzyme activity is stopped with a solution of EDTA, and the protein is denatured by heating the solution to 95 °C for 10 min, allowing the release of the aptamer and damaging at the same time all species in solution that could adsorb on the electrode surface. Finally, the released aptamers are electrochemically detected by using a gold electrode grafted with neutral PNA probes (Y1), a complementary sequence to X1. After hybridization, the previously neutral surface becomes negatively charged and

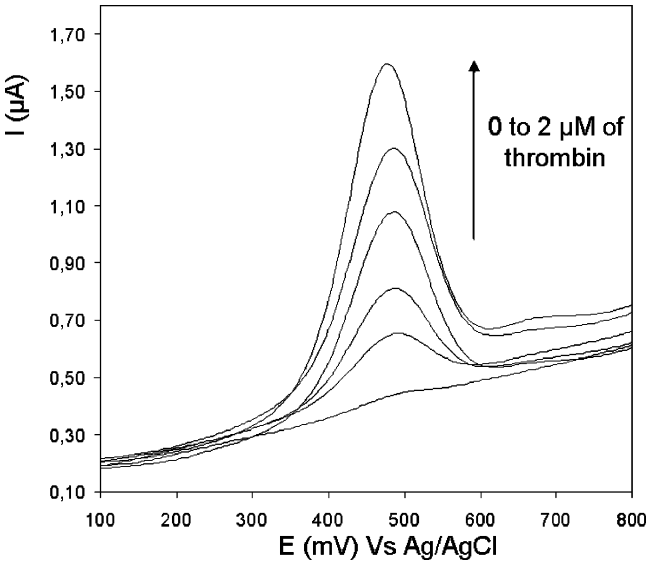

Figure 4. Square-wave voltametry responses for different human α-thrombin concentrations: 0 M, 125 nM, 250 nM, 500 nM, 1 μM, and 2 μM.

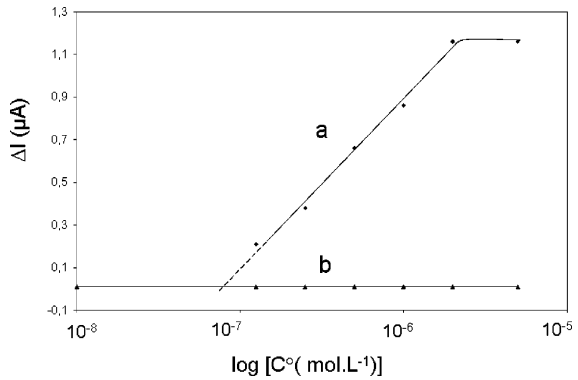

Figure 5. Differential current intensity at 490 mV (after subtraction of the blank curve) versus target concentration: (a) with solution containing aptamer X1−human α-thrombin complex and (b) with solution containing aptamer X1 and BSA. Same curve as (b) is obtained with nonspecific aptamer X2 with thrombin (not on the graph).

is revealed with the positively charged electroactive polymer 1. With this approach, the polymer only interacts with washed electrodes where only the remaining bound aptamer is present and all potential interferences are removed.

As expected, we observed a good correlation between the Fc/Fc^+ peak intensity and the protein concentration (Figure 4). The intensity increases as a function of the logarithm of the concentration of thrombin and reaches a plateau (Figure 5a) for 2 μM where all probes seem hybridized (saturation of the surface). Extrapolation seems to indicate that concentrations of ~75 nM (which means 7.5×10^{-14} mol of the protein) could be detected. To evaluate the specificity, two negative controls were carried out several times with nonspecific protein BSA or nonbinding thrombin sequence X2 (Figure 3, path b). In these conditions, no signal could be observed due to the total degradation of ss-oligonucleotides (since thrombin aptamer is not protected by BSA or sequence X2 is not bound to the thrombin protein) by S1 nuclease enzyme. In both cases, the current intensity remained unchanged compared to the blank control (Figure 5b). These results demonstrate that the indirect method is sensitive and can discriminate a specific protein from others. From all these results, it appears that the human α-thrombin-linked aptamer X1 remains intact, "protected" by the protein.

CONCLUSION

A water-soluble, electroactive, and cationic polythiophene has allowed the specific and label-free electrochemical detection of human α-thrombin by using direct (DNA probe) and indirect (S1 nuclease and PNA probe) methods. These methodologies have led a fast detection of the protein and open interesting possibilities for the future development of integrated and portable electrochemical devices for diagnostics or forensic investigations. Parallel detection of different biomolecules and an improvement of the detection sensitivity will be the subject of future investigations.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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