RNA Detection

Enzymatic Synthesis of Redox-Labeled RNA and Dual-Potential Detection at DNA-Modified Electrodes**

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Electrochemical techniques offer attractive approaches to the detection and identification of nucleic acids, a central activity of modern chemical biology. High sensitivity,[1,2] low instrumentation costs, a ready capacity for miniaturization,[3,4] and direct electronic readout offer strong motivations for the development of this field.[5–7] Various approaches have been explored, including conjugation of DNA oligonucleotides with electroactive reporters,[8–10] use of soluble electroactive mediators or intercalators,[11–14] redox enzyme mediation,[15] and measurement of direct label-free electrochemical processes[16,17] or field effects.[18,19] All of these methods rely critically upon hybridization of complementary nucleic acid sequences for specific recognition while some utilize charge transport through the π-stack of duplex DNA.[20] Of the known methods, explicit electroactive labeling has the combined advantages of a positive detection signal, a low background, and the ability to introduce several electrochemically distinguishable tags.[9,21]

The utility and fidelity of nucleic-acid-modifying enzymes such as polymerases, ligases, and nucleases is widely exploited in common genotyping approaches[22] but has been relatively underutilized in electrochemical methods of nucleic acid detection. Moreover, amplification and labeling of RNA by polymerases has been central to the development of gene expression analysis. A number of steps must be taken to integrate electrochemistry and molecular biology more effectively. Stable, enzyme-compatible, electrochemically discrete labels, robust detection interfaces, and sensitive and

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selective assay methods must all be developed. In pursuit of these goals, electrochemically labeled nucleoside triphosphates for enzymatic production of nucleic acids have recently been introduced. Electroactive deoxy-,[23–26] dideoxy-,[27] and acyclic nucleotides[26,28] have been described. Although detection of electroactive nucleic acids can be achieved with high-performance liquid chromatography or capillary electrophoresis combined with an electrochemical detector (ECD-HPLC, ECD-CE),[8,9] these transport methods are not necessarily the most suitable for miniaturized parallel analysis. Self-assembled monolayers (SAMs) of DNA oligonucleotide probes attached to a conductive substrate such as gold have been developed as electrode surfaces for nucleic acid hybridization and electrochemical analysis.[4,26,29,30] Because each electrode monolayer is analogous to a spot on a microarray, this is a good candidate system for the required level of miniaturization and parallelization.[31]

Although most electrochemical analyses have focussed upon DNA, the ease of linear amplification, high heteroduplex stability, and ready fragmentation offered by RNA are highly advantageous to analysis. RNA has previously been detected electrochemically, but earlier methods were not designed to identify specific transcripts, only bulk RNA extracts.[8,32] Other schemes have measured specific transcript levels, but only following conversion of RNA into DNA by reverse-transcriptase-PCR.[4,33] Here we describe two electrochemically distinct, enzyme-compatible ribonucleoside triphosphates ("electrotides"), which are incorporated into RNA transcripts and detected specifically at DNA-functionalized electrodes.

Ferrocene- and anthraquinone-labeled UTP derivatives ((Fc1-UTP (**1**) and Aq1-UTP (**2**), respectively, Figure 1a) were synthesized by coupling 5-(3-aminopropen-1-yl)uridine-5′-triphosphate with the carboxylic acids of the electroactive species using HATU as a condensing reagent. Yields for both triphosphate products were 20–30% after ion-exchange chromatography. Product integrity was confirmed by UV absorption, ^{1}H NMR spectroscopy, and cyclic voltammetry (CV). Fc1-UTP displayed a half-wave potential ($E_{1/2}$) of +375 mV vs Ag/AgCl with a near-ideal peak separation (ΔE_{peak}) of 75 mV. Aq1-UTP showed a reproducible quasi-reversible electrochemical behavior at a glassy carbon electrode, with $E_{1/2} = -570$ mV and $\Delta E_{peak} = 342$ mV. Free anthraquinonecarboxylate ($E_{1/2} = -460$ mV and $\Delta E_{peak} = 297$ mV) displayed a similar quasi-reversible behavior at this surface.

To determine the compatibility of **1** and **2** with the RNA polymerases conventionally employed for in vitro transcription reactions, a 232-basepair DNA template derived from the human PAI-2 coding sequence was generated by PCR. This template, which contained opposing T7 and SP6 promoter sequences for bidirectional transcription, consistently yielded a discrete major RNA transcript approximately 408 nucleotides (nt) in length from either promoter alone (Figure 1b). The predominance of this long transcript indicated efficient self-templated RNA extension of the kind described elsewhere,[34] yielding an essentially double-stranded looped RNA product (Figure 1c). Substrates **1** and **2** were similarly accommodated by T7 and SP6 RNA polymerases, displaying

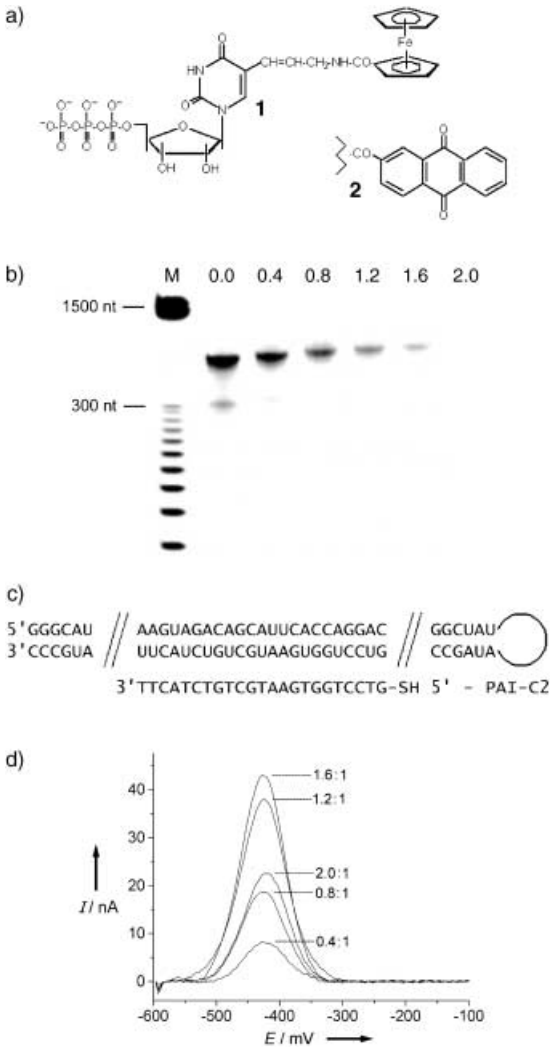

Figure 1. a) Electrotide constructs **1** and **2**. b) In vitro transcription of PAI-2 RNA at Fc1-UTP:UTP ratios between 0.0:1 and 2.0:1. Molecular weight markers (M) are indicated. c) Central region of the 408-nt PAI-2 RNA transcript with the sequence of the PAI-C2 DNA capture oligonucleotide. d) Baseline-corrected OSW voltammograms of PAI-2 RNA transcripts at various Aq1-UTP:UTP ratios.

significant incorporation over a broad concentration range (Figure 1b). Increasing the modified:unmodified UTP ratio in the transcription mixture produced more-heavily labeled transcripts as judged by the decreased electrophoretic mobility of the RNA product, an effect which is directly attributable to the higher molecular weight of the labeled nucleotide. Greater labeling was accompanied by lower RNA yields (Figure 1b), consistent with the known discrimination of phage RNA polymerases against most modified nucleoside triphosphate substrates.

For electrochemical detection of labeled RNA, self-assembled monolayers of the 5′-thiolated PAI-C2 oligonucleotide (Figure 1c) and 6-sulfanylhexanol were prepared on gold electrodes. Following hybridization of redox-labeled RNA and brief washing in PBS (phosphate-buffered saline), Osteryoung square-wave voltammetry (OSWV) of PAI-C2-derivatized electrodes produced a clear peak corresponding

to the correct electroactive moiety. Hybridization of RNA products from transcription reactions performed at several labeled:unlabeled UTP ratios yielded an optimal signal at an Aq1-UTP:UTP ratio between 1.2:1 and 1.8:1 (Figure 1d). For Fc1-UTP, the optimal ratio was very similar despite the large difference in the structures of the electroactive groups. The cause of this optimal labeled:unlabeled UTP ratio lies directly in the association of greater labeling density with lower RNA yields (Figure 1b). Subsequent experiments employed labeled:unlabeled UTP ratios within the optimal range.

To verify the specificity of the detector interface, Fc1-U-labeled RNA was also incubated with electrodes bearing the noncomplementary oligonucleotides 2A and EP. Neither noncomplementary oligonucleotide yielded a discernable signal under conditions where the complementary PAI-C2 oligonucleotide gave a strong response (Figure 2a). The detector interface is thus selective for complementary RNA sequences carrying either electrochemical moiety. To examine the concentration dependence of the interface, 1.2:1-labeled PAI-2 RNA was incubated at various concentrations with PAI-C2 electrodes. A plot of voltammogram peak area vs. RNA level (Figure 2b) appears to curve somewhat, which suggests the onset of electrode binding site saturation at high RNA levels. Approximately 200 pM RNA, equivalent to 20 fmol or 3 ng, could be reliably detected, a value that compares favorably with that of an alternative electrochemical RNA detection method.[32] In relation to the electrode surface area, 200 pM RNA is equivalent to 2.3 pmol RNA per cm^2. Because the present test system involves a worst-case hybridization where the RNA target is double stranded, we expect substantially better sensitivity with further development. Recent studies of ferrocene-modified oligonucleotides on electrode surfaces indicate that attomole sensitivities should be achievable.[35] In future studies we will investigate the sensitivity improvements to be obtained by controlling monolayer size, regularity, oligonucleotide coverage, and hybridization conditions.

In gene expression analysis, the common two-color fluorescence implementation compares the signals from treatment and control RNA samples labeled with two different fluorophores. An equivalent electrochemical implementation requires co-detection of two electrochemically distinguishable redox groups. Although different redox potentials can be produced by altering the linker or substituents on a single electroactive species, this often does not yield baseline signal resolution, requiring sophisticated AC voltammetry for signal discrimination.[9,21] As an alternative approach we have employed the two distinct electrochemical moieties of Fc1- and Aq1-UTP to achieve complete signal separation. To test this system, independent Aq1- and Fc1-RNA pools were separately transcribed from an identical template sequence at a labeled:unlabeled UTP ratio of 1.4:1. The two RNAs were mixed at different ratios and hybridized to electrodes bearing the complementary oligonucleotide probe. Characteristic signals for both the Fc1- and Aq1-labeled RNAs were observed with baseline peak separation (Figure 2c), even in the presence of large amounts of RNA. Moreover, the respective peak areas permitted to measure the relative abundance of each labeled RNA. Although the two electroactive groups were detected with slightly differing efficiencies, there was a strong linear correlation between the known Fc1-RNA:Aq1-RNA ratios applied to the electrodes and the

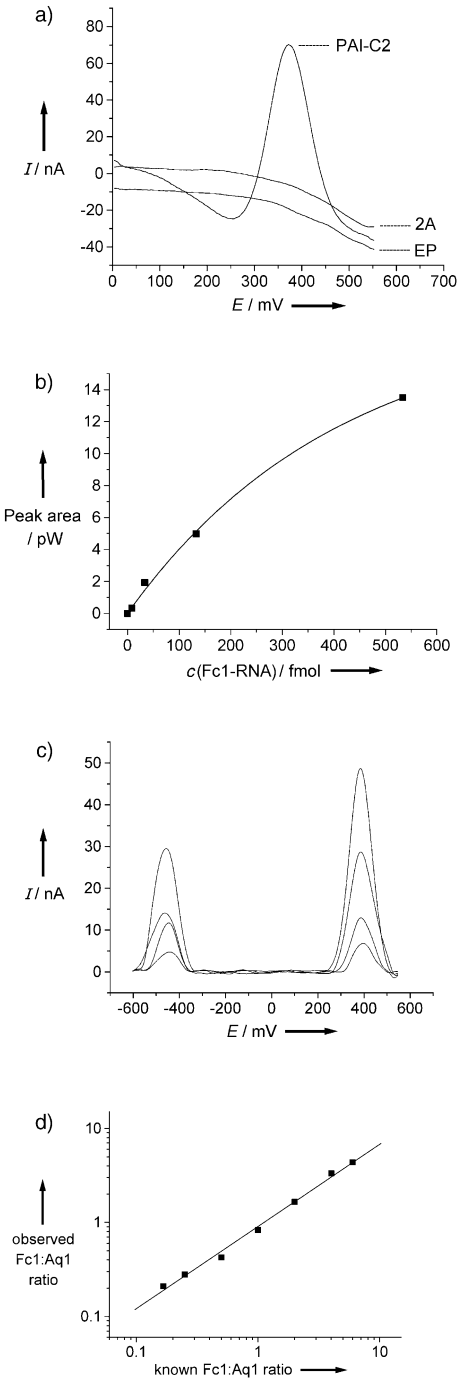

Figure 2. a) Uncorrected OSW voltammogram following incubation of Fc1-U-labeled RNA with electrodes bearing the complementary PAI-C2 oligonucleotide or the noncomplementary 2A or EP oligonucleotides. b) Target dependence of the binding of labeled PAI-2 RNA to PAI-C2-modified electrodes. c) OSW voltammograms of Fc1-U- and Aq1-U-labeled RNAs co-hybridized to electrodes. Traces derived from Fc1-RNA:Aq1-RNA ratios of 6:1, 2:1, 1:2, and 1:6 are shown. d) Comparison of known Fc1-RNA:Aq1-RNA ratios applied to electrodes with observed Fc1:Aq1 OSW voltammogram peak area ratios. The line of best fit is shown.

OSW voltammogram peak area ratios (Figure 2d), indicating that these two groups are compatible with RNA quantitation.

We have described the direct, specific and quantitative detection of electroactive RNA in an electrode-based format amenable to miniaturization and parallelization. The use of readily synthesized, polymerase-compatible, electrochemically distinct electrotides broadens the application of electrochemical detection methods to nucleic acids. With further development of nucleotides, interfaces, and detection methods, we expect this approach to be useful not only for the co-detection of same-sequence transcripts, but also to the development of electrochemical RNA biosensors for a variety of applications including detection of single nucleotide polymorphisms (SNPs) and genome identification.

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