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COMMUNICATIONS

Published on Web 03/13/2004

Multipotential Electrochemical Detection of Primer Extension Reactions on DNA Self-Assembled Monolayers

Daniel A. Di Giusto,[†] Wjatschesslaw A. Wlassoff,[†] Susanne Giesebrecht,[†] J. Justin Gooding,[‡] and Garry C. King*,[†]

School of Biotechnology and Biomolecular Sciences and School of Chemical Sciences, The University of New South Wales, Sydney, New South Wales 2052, Australia

Received December 22, 2003; E-mail: garry@kinglab.unsw.edu.au

Electrochemical detection of nucleic acids is an attractive alternative to established mass- and fluorescence-based methodologies, with advantages including cost, sensitivity, and direct electronic readout.[1] The development of self-assembled monolayers (SAMs) of DNA oligonucleotides on gold electrodes[2−6] has facilitated assay development, coupling the specificity of nucleic acid hybridization to a bioelectronic interface. Target nucleic acids can be captured on these DNA SAMs, especially for single-nucleotide polymorphism (SNP) identification. One potential SNP genotyping strategy has relied upon differential charge conduction from electroactive intercalators through matched or mismatched duplexes to the SAM electrode surface.[7,8] The capture of labeled electroactive probes near the SAM surface has also been described.[9,10] While these methods use Watson−Crick hybridization to discriminate between very similar nucleic acids, it should also be possible to achieve more robust identification by utilizing the intrinsic fidelity of enzymes. With these applications in mind, electroactive nucleoside triphosphates have recently been introduced for incorporation by polymerases or terminal transferases.[11−13] These species have first been applied in HPLC-ECD,[12] in capillary gel electrophoresis (CE)-based primer extension,[14−16] and in the detection of viral DNA following redox enzyme amplification.[17] In this communication we describe a range of electroactive nucleoside triphosphates ("electrotides") and their use in polymerase-mediated single base extension (SBE) assays with specific detection on DNA SAMs in several alternative formats.

Electrotides bearing three different electroactive groups from two chemical classes were prepared (Figure 1). Ferrocene-labeled dUTP was synthesized in two forms differing in the linker between the nucleobase and the electroactive moiety, designated Vinyl-Fc-dUTP (**1a**) and Fc1-dUTP (**1b**). These species display half-wave potentials $E_{1/2} = +169$ and $+376$ mV vs Ag/AgCl, respectively. An anthraquinone-based electrotide was produced through a similar conjugation reaction with dCTP, forming Aq1-dCTP (**2**) with $E_{1/2} = -551$ mV. In addition, a terminator based on acyclo sugar chemistry and linked with a ferrocene moiety, Fc1-acyUTP (**3**) with $E_{1/2} = +344$ mV, was synthesized and purified. The integrity of all constructs was confirmed by UV absorption, ^{1}H NMR, ESI-MS, and cyclic voltammetry.

In the first implementation, SBE primer extension reactions in solution were performed with exo$^-$ A488L Vent DNA polymerase, a mutant enzyme capable of incorporating both dNTPs and acyNTPs.[18] Polymerase was incubated with Vinyl-Fc-dUTP (**1a**), oligonucleotide primer and template containing either an "A" (wildtype) or "C" (mutant) nucleotide polymorphism at the position directly downstream from the 3′ primer terminus (Figure 2A). SBE reaction products were purified by gel filtration chromatography

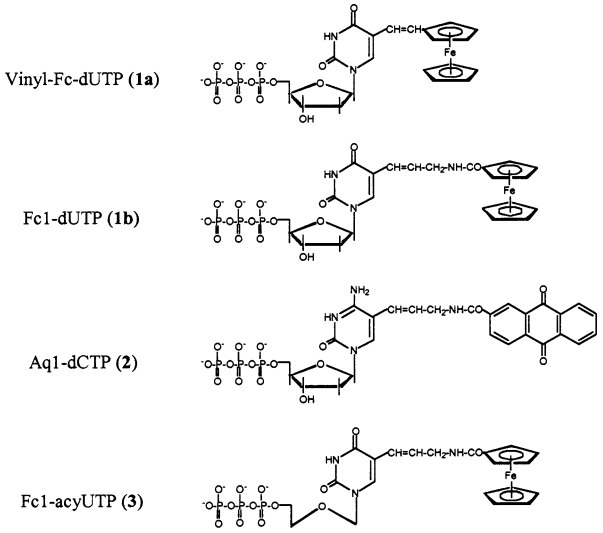

Figure 1. Electroactive nucleoside triphosphates (electrotides) for polymerase-mediated primer extension reactions.

and incubated with DNA SAMs constructed by immobilizing thiolated oligonucleotides (typical oligonucleotide density 9×10^{12} molecules/cm^2 as measured by the ruthenium hexammine method[19]) in a 6-mercapto-1-hexanol passivation layer.

Three alternative DNA SAM formats were employed for capture of solution SBE products (Figure 2B): a generic antitag 20mer sequence immobilized at the 5′ end (AT5 format), a 20mer antitag immobilized at the 3′ end (AT3), or a perfect primer complement (COM) immobilized at the 5′ end. In a fourth and unique detection format, template DNA was hybridized to a DNA SAM followed by direct surface extension (DSE) of the SAM oligonucleotide by polymerase (Figure 2B).

All four detection formats yielded distinct electrochemical signals for incorporated Vinyl-Fc-dU by Osteryoung square wave voltammetry (OSWV), indicating the utility of this general approach (Figure 2C). Experimental $E_{1/2}$ values observed on the SAM were closely consistent across the four detection formats. Interestingly, direct surface extension consistently yielded a stronger OSWV signal than the other capture formats (Figure 2C), a point for future exploration.

To further examine the question of polymerase-mediated specificity, direct SAM-primer extension (DSE) was performed using each of the four electrotides and alternative polymorphic templates. Distinct OSWV signals were readily observed for the different electroactive moieties at near-baseline resolution (Figure 3A). Notably, anthraquinone-bearing Aq1-dCTP (**2**) was incorporated and detected with an efficiency near that of the ferrocene-bearing electrotides (Figure 3A, trace a). To assess the quantitative

[†] School of Biotechnology and Biomolecular Sciences.
[‡] School of Chemical Sciences.

10.1021/ja0319036 CCC: $27.50

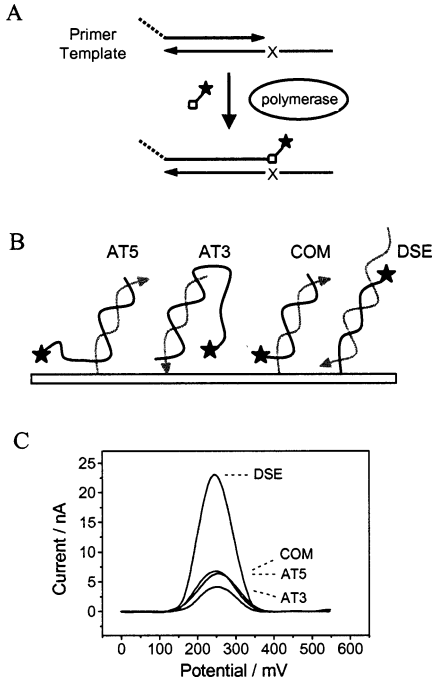

Figure 2. (A) SBE of a primer oligonucleotide with an electroactive nucleoside triphosphate. The primer tag sequence is dotted. (B) Alternative formats for capture of solution-extended primers (AT5, AT3, and COM) or for direct surface extension (DSE) of primer. (C) Osteryoung square wave voltammograms (OSWV) of Au−DNA SAM electrodes following capture or extension of primer oligonucleotides with Vinyl-Fc-dUTP (**1a**) in the different formats of Figure 2B.

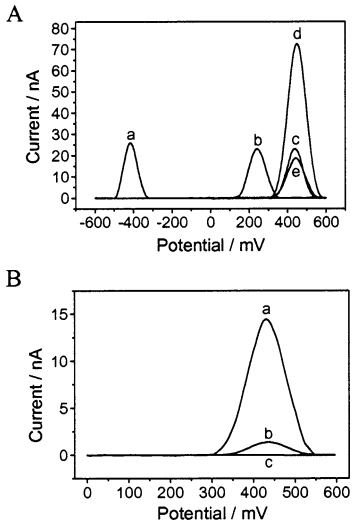

Figure 3. (A) Combined OSWV scans of DNA SAMs following direct extension with the range of electrotides: (a) Aq1-dCTP (**2**) with single-G template, (b) Vinyl-Fc-dUTP (**1a**) with single-A template, (c) Fc1-dUTP (**1b**) with single-A template, (d) Fc1-dUTP (**1b**) with multi-A template, and (e) Fc1-acyUTP (**3**) with single-A template. (B) OSWV scans of DNA SAMs following extension by Fc1-acyUTP (**3**): (a) Correct ("A") template, (b) Incorrect ("C") template, and (c) wild-type template without enzyme addition.

performance of this system, use of the nonterminating Fc1-dUTP (**1b**) electrotide with a template strand containing a string of four A nucleotides downstream from the 3′ primer terminus yielded an electrochemical peak area 3.7× that of the normal single A template (Figure 3A, traces c and d). This performance is not suitable for allelic quantitation but is adequate for biallelic systems.

The accuracy of template polymorphism identification was determined with the terminator electrotide Fc1-acyUTP (**3**) in a direct SAM-primer extension with either wildtype (correct) or mutant (incorrect) template DNA (Figure 3B). A strong OSWV peak was observed for the correct template, while incorporation of the electrotide against the incorrect C template was minimal, with a peak area signal-to-noise ratio of 10.9:1 for correct-to-incorrect extension. This initial performance is encouraging and is likely to be further improved by optimization of the SAM interface and possibly by the introduction of a proofreading DNA polymerase and suitably modified primer.[20]

We have described a range of electroactive nucleoside triphosphates compatible with primer extension reactions that display signals easily distinguishable by electrochemical methods such as OSWV. DNA self-assembled monolayers on gold electrodes have been used as the detection platform, with several alternative extension and capture formats demonstrated. Moreover, the SBE reaction is shown to detect the identity of a model single-nucleotide polymorphism with high accuracy. The development of electrochemically distinguishable nucleotides in combination with a sensitive, accurate, and parallelizable detection platform may open the way for more widespread electrochemical genotyping of nucleic acids.

Acknowledgment. This work was supported in part by ARC Grant LP0347371. We thank Bill Jack (New England Biolabs) for the gift of DNA polymerases, Russell Pickford and Michael Guilhaus for assistance with ESI-MS, and Barbara Messerle for discussions.

Supporting Information Available: Electrotide syntheses, NMR and CV data; oligonucleotide sequences; electrode and SAM construction; extension reaction protocols; SAM incubation and electrochemical measurements (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0319036