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Electrochemical detection of modified maize gene sequences by multiplexed labeling with osmium tetroxide bipyridine

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ABSTRACT

In this report we demonstrate an approach for the electrochemical detection of four sequences from maize and genetically modified (GM) maize by means of square-wave voltammetry (SWV). After multiplexed labeling with osmium tetroxide bipyridine ([OsO_4(bipy)]), the target oligonucleotides are hybridized with a complementary DNA capture probe immobilized on gold electrodes. The multiplexed labeling was performed by mixing the four target strands with the respective oligonucleotides 80% homologous to the central target recognition sequences in order to protect the latter from binding of [OsO_4(bipy)] to its thymine or cytosine residues. All components were added to the same solution. No significant decreases in SWV hybridization signals were observed after such multiplexed labeling of up to four target strands in the same reaction batch. Obtained voltammetric signals were significantly higher at 50 °C compared to 25 °C hybridization temperature and very low response was observed for non-complementary strands. Multiplexed labeling with osmium tetroxide bipyridine holds great promise for the development of simple and effective voltammetric detection protocols for GM organisms.

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1. Introduction

Over the past decade, electrochemical detection of nucleic acid hybridization has become a common tool besides classical molecular biological methods such as Southern or Northern blots. The electrochemical transduction can be performed using covalent labels, intercalators or ions interacting electrostatically with the DNA. Exploiting the electrochemical activity of the DNA is also possible.

Electrochemical detection of DNA sequences can be performed such as by using self-assembled monolayers of capture probes on electrode surfaces [1–6] or hybridization at magnetic beads coupled with AdSV ("two surface strategy") [7,8]. Our group reported about [OsO_4(bipy)] as an electrochemically reversible covalent DNA-label together with thiol-linked probe oligonucleotides on gold electrodes [9].

Other approaches for the electrochemical hybridization detection of PCR products or oligonucleotides include methylene blue [10,11] or sandwich hybridization with an alkaline phosphatase conjugate on biotinylated signaling probes [12]. The different methods of electrochemical hybridization detection have been reviewed several times [13–18]. The complex [OsO_4(bipy)] can be utilized as an electrochemically reversible covalent DNA-label as described by Palecek et al. [19–22]. It reacts with the pyrimidine bases [23] under oxidation of the C–C-double bond in the pyrimidine ring forming a diester of osmic(VI) acid. This modification can be easily accomplished with single-stranded oligonucleotides or PCR products by formation of a central double strand with a protective partly homologous oligonucleotide as we have described earlier [9,25,26]. Besides single-stranded DNA, also tRNA can be labeled with osmium tetroxide [27]. Also PNA can be modified with this osmium complex, allowing stripping detection at the low pM level [24]. The temperature influence upon the hybridization process using protective strands has been characterized earlier [28]. "Multicolor labeling" of different target strands is possible using osmium tetroxide complexes with different tertiary amine ligands, which influence the electrochemical potential of the resulting covalent osmium(VI)-labels [29].

Here, we describe a simple approach for multiplexed covalent labeling of target oligonucleotides representing four sequences designed to discriminate between natural and genetically modified maize.

2. Experimental

2.1. Material

2,2′-Bipyridine was delivered by Merck and osmium tetroxide was obtained as a 2% aqueous solution from Fluka. All DNA

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oligonucleotides (*probes, targets and protector strands*) were delivered by Friz Biochem (Neuried, Germany). The "tris-buffer" contained 10 mmol/l tris-(hydroxymethyl)-aminomethane and 0.5 mol/l sodium sulfate and was adjusted to pH 7.5 using sulfuric acid. The sequences of all DNA-strands together with their length and designations are given in Table 1. All *protector* oligonucleotides contain 3 to 5 mismatches (underlined in Table 1) to ease their later displacement by the immobilized probes.

2.2. Modification of target oligonucleotides

The *target oligonucleotides* and the 80% homologous protective strands were mixed in equimolar ratio and left for two hours at room temperature to allow hybridization. The probe SSIIb detects the starch synthase gene IIb and the probe ivrp detects the invertase gene of maize therefore serving as a positive control for the detection of maize. The probe CRY detects the existence of the cryIa/b transgene within the sample and the probe 810 detects the existence of the transgene at the MON 810 specific insertion locus in the maize genome. By choosing these sequences one can (a) detect the presence of maize (SSIIb and ivrp), (b) detect the presence of a specific transgene cryIa/b (CRY) and (c) detect the presence of the specific event Mon 810 (810) [30]. The sequences are given in Table 1.

The dialysis procedure, the preparation of SAM-modified gold electrodes, the hybridization and dehybridization procedures, and the electrochemical measurements have been described in more detail earlier [9].

3. Results and discussion

Fig. 1A and B display the effect of the hybridization temperature upon SWV signals of the capture probe ivrp hybridized with either the complementary ivrp target or the non-complementary 810 target. In this figure as in all other figures where hybridization signals are displayed as voltammetric responses, representative peaks were chosen that were closest to the average value of three individual experiments. While the response of both strands is visible after room temperature hybridization (Fig. 1A), the signal of ivrp is about 10-times higher than the non-complementary strand. At optimized 50 °C hybridization temperature, the non-specific response is completely suppressed (Fig. 1B). Similar optimized conditions were used in previous work [26]. This demonstrates not only the selectivity of the probes to their respective targets (as it is displayed further in Fig. 2), but also the influence of optimized temperature. The latter does not only increase the selectivity, but also the sensitivity of the sensor. We found the signal height to be approximately four times higher at 50 °C compared to room temperature. Further temperature optimization is displayed also in Fig. 1 (C and D). It depicts calibration plots for the targets 810 and ivrp at 40 °C (C) and 50 °C (D) hybridization temperature in three separate calibration experiments each with separately prepared target, probe and electrode. 10 nM target could be detected in both cases. The signal increase when going from 40 °C to 50 °C was 2-fold and 4-fold in case of 810 and ivrp, respectively. The lower signal of ivrp at 40 °C is probably due to stable secondary structures of the probe ivrp at lower temperatures. Other reasons for increased signals at elevated hybridization temperature include enhanced mass transport due to diffusion and thermal microstirring [31]. At 40 °C, linear calibration ranges could be found up to 50 and 100 nM for 810 and ivrp, respectively (Fig. 1C). At 50 °C, however, an almost linear calibration range could be observed between 25 and 200 nM for both targets (Fig. 1D). Although coefficient of determination r^2 was better for a squared calibration function compared to a linear one, the difference between the residual sums of squares was not significant (*F*-test, not shown). These findings indicate how important an individually optimized hybridization temperature is. Reproducibility (as indicated by error bars) was found to be good even if comparing responses of independent repetitive measurement series each obtained with a newly prepared probe SAM. Similar high standard deviations have been observed before [26].

Fig. 2 exhibits how the four different capture probes ivrp, SSIIb, 810 and CRY (Table 1) respond to the four different targets in separate experiments. In Fig. 2A, the capture probe ivrp yields only response after hybridization with the full matching target ivrp. All other sequences are non-specific and fully suppressed. In comparison, the three other capture probes show full selectivity only with certain non-specific strands; however, in all cases, the non-specific response is greatly reduced due to the optimized 50 °C hybridization temperature. In these cases, all targets had been protected with their special protective strands during the modification with osmium tetroxide bipyridine.

In Fig. 3, it is demonstrated how a sample containing different target strands can be investigated using different capture probe-modified electrodes. Error bars indicate standard deviations of three independent repetitive experiments with different targets, probes and newly prepared probe SAMs. Probe ivrp only yields large response when the mixed sample contains the ivrp target sequence (Fig. 3A). Mixed samples containing mixed non-specific 810, SSIIb and CRY sequences yield much smaller signals (10 to 30 times smaller). Similar applies to probe 810 as displayed in Fig. 3B. Only target 810 gave a large response while signals of the three mixed non-specific sequences have been greatly suppressed.

Furthermore, we demonstrate in Fig. 3C how genetically modified maize sequences can be discriminated from the wild type sequence by means of four working electrodes, each modified with another kind of capture probes. Probe 810 and probe CRY, which are designed for indicating the genetic modification, yielded only

Table 1
Sequences of deoxyoligonucleotides.

Designation	Length	Sequence
Probe ivrp	29 + 15 a	5′-CACGTGAGAATTTCCGTCTACTCGAGCCT- aaaaaaaaaaaaaaa[Dithio]3-3′
Target ivrp	29 + 5 t	5′-tttttAGGCTCGAGTAGACGGAAATTCTCACGTG-3′
Protector ivrp	29	5′-CAGCTGAGAATTTCCGTCTAGTGCAGCCT-3′
Probe SSIIb	25 + 15 a	5′-AGCAAAGTCAGAGCGCTGCAATGCA-aaaaaaaaaaaaaaa[Dithio]3-3′
Target SSIIb	25 + 5 t	5′-tttttTGCATTGCAGCGCTCTGACTTTGCT-3′
Protector SSIIb	25	5′-ACGAAAGTCAGAGCGCTCGAATGGA-3′
Probe CRY	26 + 15 a	5′-AGATACCAAGCGGCCATGGACAACAA- aaaaaaaaaaaaaaa[Dithio]3-3′
Target CRY	26 + 5 t	5′-tttttTTGTTGTCCATGGCCGCTTGGTATCT-3′
Protector CRY	26	5′-AGATAGCAAGCGGCCATGCAGAAGAA-3′
Probe 810	23 + 15 a	5′-AACATCCTTTGCCATTGCCCAGC- aaaaaaaaaaaaaaa[Dithio]3-3′
Target 810	23 + 5 t	5′-tttttGCTGGGCAATGGCAAAGGATGTT-3′
Protector 810	23	5′-AACATCCTTTGGCATTCCCCACG-3′

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