

Full Research Paper

Enzyme-Linked Electrochemical Detection of PCR-Amplified Nucleotide Sequences Using Disposable Screen-Printed Sensors. Applications in Gene Expression Monitoring

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Abstract: Electrochemical enzyme-linked techniques for sequence-specific DNA sensing are presented. These techniques are based on attachment of streptavidin-alkaline phosphatase conjugate to biotin tags tethered to DNA immobilized at the surface of disposable screen-printed carbon electrodes (SPCE), followed by production and electrochemical determination of an electroactive indicator, 1-naphthol. Via hybridization of SPCE surface-confined target DNAs with end-biotinylated probes, highly specific discrimination between complementary and non-complementary nucleotide sequences was achieved. The enzyme-linked DNA hybridization assay has been successfully applied in analysis of PCR-amplified real genomic DNA sequences, as well as in monitoring of plant tissue-specific gene expression. In addition, we present an alternative approach involving sequence-specific incorporation of biotin-labeled nucleotides into DNA by primer extension. Introduction of multiple biotin tags per probe primer resulted in considerable enhancement of the signal intensity and improvement of the specificity of detection.

Keywords: electrochemical detection; enzyme-linked assay; DNA hybridization; primer extension; PCR; gene expression

1. Introduction

Labeling of biomolecules with enzymes has been widely applied in various bioassays due to the advantages of the inherent “biocatalytic” signal amplification. Each enzyme molecule, tethered to an antibody, a nucleic acid probe or other recognition element, can convert many molecules of a suitable substrate into detectable indicator product. This renders the enzyme-linked assays considerably sensitive. The immunochemical technique well known as ELISA [1-3] belongs to the core methodology of analysis of proteins and other biologically important species. Colorimetric or chemiluminescence-based techniques employing enzyme-labeled antibodies have successfully been applied, for example, in studies of proteins posttranslation modifications [4], DNA-protein interactions [3, 5], gene expression [1], etc. Electrochemical enzyme-linked immunoassay techniques have also been reported [6-10].

Electrochemical DNA hybridization techniques involving enzyme-labeled probes have recently been proposed as well [7, 8, 11-18]. For electrochemical detection, the marker enzyme is required to catalyze conversion of an inactive substrate into electrochemically active or surface-active indicator which can subsequently be detected by voltammetry [13, 14], amperometry [12], impedance spectroscopy [16] or other technique. For instance, alkaline phosphatase (ALP) has been applied [8, 13-15] in connection with phosphor-esters of phenols such as 1-naphthol or p-aminophenol. The phenol phosphates are electrochemically inactive while the parent phenols, released from the esters by the ALP, are electrochemically oxidizable. Hence, the DNA hybridization events can be detected via measurements of the released phenol signals which appear only in the presence of the enzyme tag. Besides ALP, other enzymes such as peroxidases [11, 12] or β -galactosidase [17], in connection with suitable substrates, have been applied in electrochemical DNA hybridization assays.

Enzyme-linked DNA sensing techniques have been applied in various experimental arrangements. The ELISA microwells [11] or different types of magnetic beads [8, 14, 15, 17] have been utilized as solid substrates for immobilization of capture probes and/or the target DNAs (tDNA) and performing hybridization of the tDNAs with the enzyme-labeled reporter probes. In other approaches, the capture probes or tDNAs were attached to the detection (transducer) electrode and both DNA hybridization and electrochemical detection were conducted at the same surface [12, 13]. Using thermostable soybean peroxidase, a sensor for mismatch-sensitive enzyme-amplified detection of DNA hybridization was proposed [12]. Another approach, based on hybridization between tDNAs adsorbed at carbon electrodes and ALP-labeled signaling probes, has recently been proposed [13] for the determination of trinucleotide repeat lengths in polymerase chain reaction (PCR)-amplified genomic DNA fragments.

In the DNA hybridization experiments, the enzymes have often been coupled to the probe via biotin-(strept)avidin linkage, i.e., commercially available (strept)avidin-enzyme conjugates were attached to a biotinylated nucleic acid. Employment of the biotin-(strept)avidin technology offers utilization of an alternative approach, based on incorporation of the biotin-labeled nucleotides into DNA using DNA polymerases (instead of hybridization between tDNA with a biotinylated probe). Primer extension (PEX)-based assays involving labeled deoxynucleotide triphosphates (dNTPs) are routinely used in modern DNA sequencing techniques [19, 20] and have been applied also in connection with the electrochemical detection platform [16, 21-25]. Besides ferrocene-dNTP

conjugates most frequently used for these purposes [21-24], other electrochemically active moieties such as nitro- or amino phenyl derivatives have recently been coupled to dNTPs and incorporated into DNA [25]. PEX incorporation of biotinylated dNTPs, followed by attachment of enzymes producing electrochemically detectable indicators, has also been reported [16].

In this paper, applications of simple enzyme-linked electrochemical techniques, in connection with disposable screen-printed carbon electrodes (SPCE), for the detection of non-repetitive genomic DNA sequences amplified by PCR are proposed. The detection system involves biotin DNA labels, ALP-streptavidin conjugate (SALP) and 1-naphthyl phosphate as a substrate to be converted into the 1-naphthol indicator. We show an excellent differentiation between complementary and non-complementary DNA sequences via hybridization of tDNA adsorbed at SPCE with biotinylated probes, as well as highly specific detection of PCR-amplified genomic DNA fragments by means of a newly introduced PEX-based assay. In connection with a reverse transcription-PCR (RT-PCR) technique, application of the enzyme-linked electrochemical assay in gene expression monitoring is demonstrated.

2. Results and Discussion

It has been reported previously that some types of carbon electrodes (such as carbon paste [26, 27], graphite-composite [18], pyrolytic graphite or screen-printed electrodes [13]) can be used as substrates for DNA hybridization without any special surface modifications, interfacing and/or covalent immobilization of capture probes (reviewed in [28, 29]). Physisorbed single-stranded (ss) DNA (or peptide nucleic acid [26]) was shown to be able of forming duplex with complementary DNA strands in solution to which the ssDNA-modified electrode was exposed. Sensors based on probe or tDNA adsorption at carbon electrodes have been combined with various detection principles, including label-free detection employing intrinsic DNA electroactivity [30], application of non-covalent redox indicators (such as $[\text{Co}(\text{phen})_3]^{3+/2+}$, methylene blue [27], Meldola's blue [31] or others) or probes labeled with enzymes [13, 18].

Here we applied an enzyme-linked voltammetric technique, proposed previously for hybridization analysis of repetitive DNA sequences [13], to detect hybridization between non-repetitive, random-sequence tDNAs and complementary biotinylated probes at the SPCE surface. Figure 1A shows scheme of the experiment. The tDNA was adsorbed at the SPCE at open current circuit from a small (6- μl) aliquot of the sample. When the Gwent C 10903P14 ink[†] was used for the SPCE preparation, no pretreatment of the electrode prior to DNA adsorption was necessary to obtain well defined and reasonably reproducible responses. After adsorption of the tDNA, the unoccupied electrode surface was blocked by bovine serum albumin, followed by subsequent application of the biotinylated probe solution and the SALP conjugate solution. Blocking of the electrode prior to the hybridization step was critical for specificity of the sensor responses: when it was omitted or performed after incubation with

[†] Besides C 10903P14, we tested several other Gwent inks. While inks C2050617 D2, C2030519 and C2010517 D4 exhibited more or less similar properties when used in the presented enzyme-linked DNA sensing techniques, C2050517 D1 and C50905 D1 appeared unusable for the same purpose. More details about the SPCE preparation and evaluation of different kinds of carbon inks will be published elsewhere.

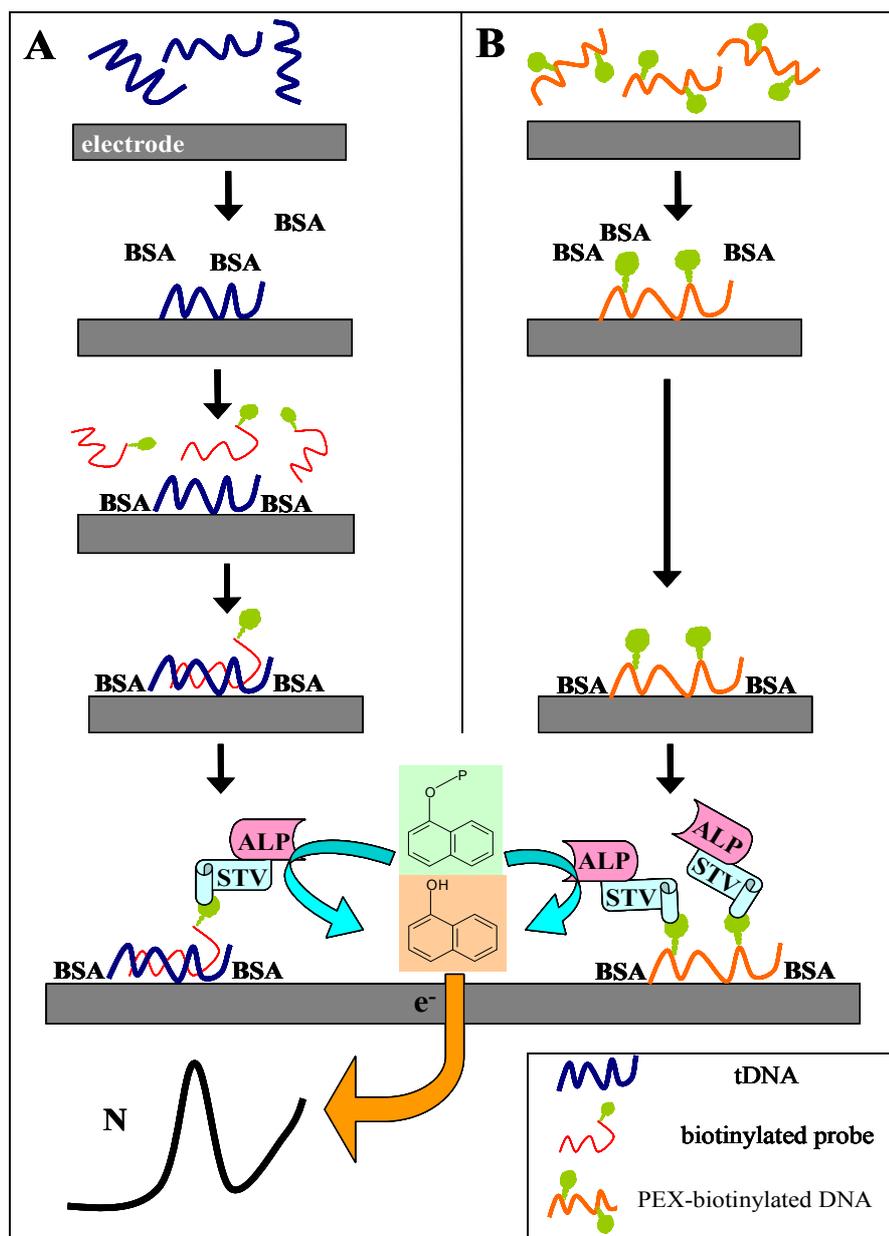


Figure 1. Scheme of the electrochemical enzyme-linked DNA sensing procedures. **(A)** Hybridization of unlabeled target DNA (tDNA) with biotinylated probe at the electrode surface. The tDNA is adsorbed at the screen-printed carbon electrode (SPCE). Unoccupied electrode surface is blocked by bovine serum albumin (BSA), followed by hybridization of the tDNA with biotinylated probe and binding of streptavidin (STV) alkaline phosphatase (ALP) conjugate to the probe biotin tags. After washing, the SPCE is dipped into background electrolyte solution containing 1-naphtyl phosphate. This substrate is enzymatically converted into an electroactive indicator, 1-naphthol, that is subsequently detected via its electrochemical oxidation. **(B)** Detection of biotin tags incorporated into probe DNA by primer extension (PEX). The PEX product is adsorbed at the SPCE, followed by electrode blocking, SALP binding, indicator production and electrochemical detection.

the biotinylated probe, false positive responses were obtained due to unspecific adsorption of the probe at the electrode surface [13]. Finally, the electrode was dipped into solution of the substrate (1-naphthyl phosphate) in background electrolyte, and after a short incubation time during which the substrate was enzymatically converted into the electroactive indicator (1-naphthol), signal of the latter was measured using linear sweep voltammetry (LSV).

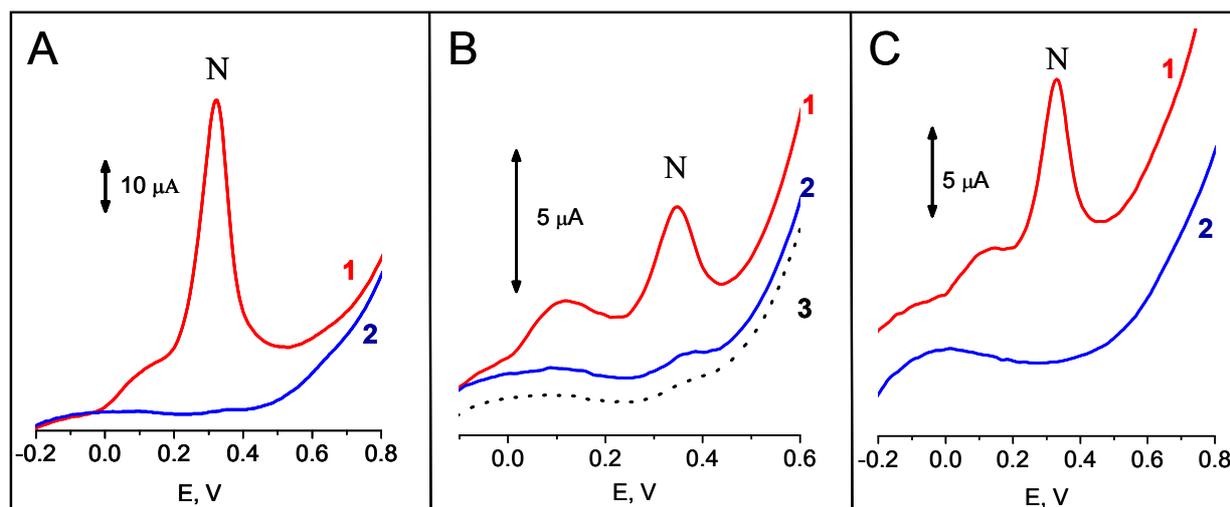


Figure 2. Typical responses resulting from the electrochemical enzyme-linked assays. (A) DNA hybridization with synthetic 40-mer target ODNs. Target T^{p53} ($5 \mu\text{g mL}^{-1}$) was adsorbed at the electrode surface, followed by electrode blocking and incubation with biotinylated probes ($5 \mu\text{g mL}^{-1}$): (curve 1), complementary P^{p53} probe; or (curve 2), non-complementary P^{rbcl} probe. Procedure shown in Fig. 1A was used. (B) As in (A) but PCR-amplified tDNA fragment fr^{p53} was used instead of the T^{p53} ODN; curve 3 corresponds to negative control (no tDNA adsorbed). The PCR product was adsorbed at the electrode from denaturing medium to achieve separation of its complementary DNA strands. (C) Responses to (curve 1), a biotinylated ODN p^{p53} ($0.4 \mu\text{M}$); or (curve 2), $dU^{bio}TP$ (5 mM). In this case, procedure shown in Fig. 1B was used. Peak N is due to electrochemical oxidation of 1-naphthol; for more details, see Experimental Section.

2.1 Hybridization with synthetic oligonucleotides

Typical hybridization response obtained for a model tDNA T^{p53} (Table I), a synthetic 40-mer oligonucleotide (ODN) involving nucleotide sequence derived from coding part of human tumor suppressor gene *p53* [3-5], is shown in Figure 2A. The target ODN was adsorbed at the SPCE from $5 \mu\text{g mL}^{-1}$ solution in 0.3 M NaCl , 10 mM Tris-HCl , pH 7.6 (buffer H) and hybridized with the complementary P^{p53} probe (a 20-mer ODN bearing biotin tag at its 3'-end; $5 \mu\text{g mL}^{-1}$ in the buffer H) for 120 s. Incubation of the sensor in the substrate solution prior to the voltammetric measurement took 60 s. Under these conditions, a large peak N due to electrochemical oxidation of 1-naphthol at

about +0.32 V was detected (Fig. 2A). When P^{rbcl} probe was used instead of P^{p53} , only negligible peak N was observed, in agreement with a lack of complementarity between the T^{p53} target and the P^{rbcl} probe (Fig. 2A). Similarly, when T^{p53} was replaced by an ODN NT^{gtt7} , no significant signal was observed after incubation with the P^{p53} (Fig. 3) or P^{rbcl} (not shown) probes. Hence, an excellent discrimination between ODNs complementary and non-complementary to the biotinylated probe was attained using the enzyme-linked electrochemical DNA hybridization assay.

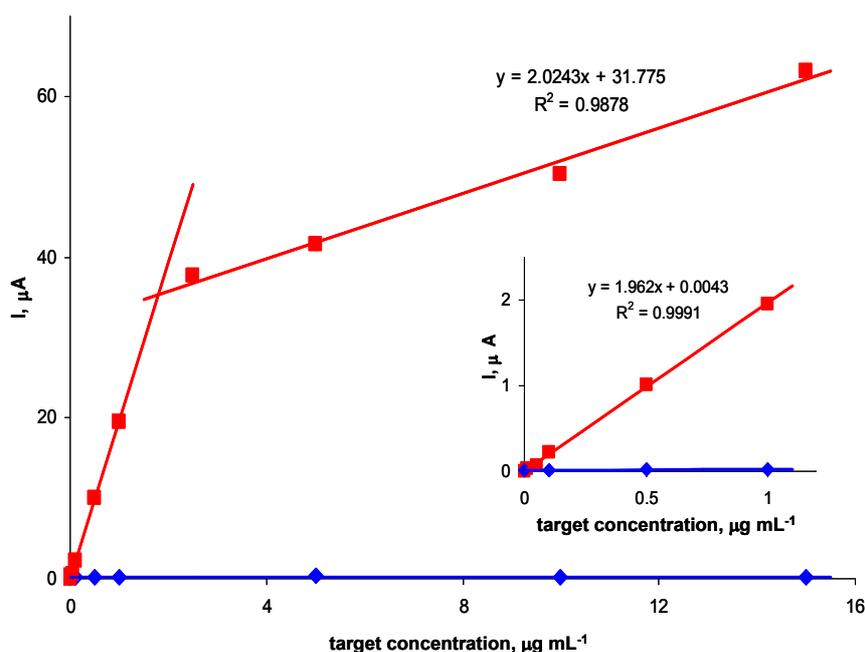


Figure 3. Dependence of the peak N height on concentrations of the target ODNs: (red), T^{p53} ; (blue), NT^{gtt7} . The experiment was performed as in Fig. 2A using tDNA concentrations given in the graph and the P^{p53} probe at a constant concentration of $5 \mu\text{g mL}^{-1}$. *Inset*; detail of the tDNA concentration dependence in the range from 0 to $1 \mu\text{g mL}^{-1}$.

We further studied effects of tDNA concentration on the peak N intensity (Fig. 3). For the T^{p53} target (applied at concentrations varying between 0 and $15 \mu\text{g mL}^{-1}$) and P^{p53} (applied always at a concentration of $5 \mu\text{g mL}^{-1}$), a biphasic dependence of the signal intensity on tDNA concentration, consisting of two linear segments, was observed. Between 0 and $\sim 5 \mu\text{g mL}^{-1}$ the peak N height increased steeply with increasing T^{p53} concentration, followed by a region of less steeply increasing signal. Such shape of the dependence suggested saturation of the electrode surface by the tDNA molecules. In addition, other phenomena such as sterical clashes affecting the hybridization process (and/or SALP binding) at high surface concentrations of the tDNA [28, 29], may contribute to the observed break on the tDNA concentration-signal intensity dependence. Under the given conditions, the T^{p53} target ODN was detectable (reliably distinguishable from the non-specific ODN NT^{gtt7}) down to 50 ng mL^{-1} (corresponding to 300 pg in a 6- μl sample) i.e., about 3.8 nM (22 fmol in 6 μl). With the non-complementary NT^{gtt7} , only the negligible background signal was observed for any tDNA concentration between 0 and $16 \mu\text{g mL}^{-1}$.

2.2 Hybridization with PCR-amplified genomic fragments

In contrast to the model synthetic ODNs, PCR-amplified DNA fragments are inherently double-stranded. Hence, denaturation of the duplex PCR product prior to adsorption at the electrode surface is necessary to make hybridization between the biotinylated probe and complementary stretch within one of the amplicon strands possible. Previously we showed [13] that tDNA can be adsorbed at the carbon electrode surface from denaturing medium (20 mM NaOH) without loss of its hybridization capacity. The efficacy of tDNA hybridization with the signaling probe (performed in neutral medium) was under such conditions even higher, when compared to results obtained with thermally denatured tDNAs adsorbed at the electrode from physiological media, due to prevention of undesired renaturation of the amplicon duplexes. Here we used an analogous procedure and adsorbed the PCR products at the SPCE from solution containing 20 mM NaOH and 180 mM NaCl; other steps of the analytical protocol were performed as with the model target ODNs.

Figure 2B shows responses resulting from hybridization of a PCR-amplified 347-bp fragment of *p53* cDNA, **fr^{p53}**, with probes **P^{p53}** (complementary) or **P^{rbcl}** (non-complementary). For the **P^{p53}** probe, a well defined peak N was detected, while for the **P^{rbcl}** probe only the small background signal was observed. When the **P^{rbcl}** probe was hybridized with **fr^{rbcl}**, a 264-bp amplicon of the *rbcl* cDNA possessing a stretch complementary to the **P^{rbcl}**, the resulting response was similar to that observed for the **fr^{p53}** - **P^{p53}** pair (see Fig. 4). The presented electrochemical enzyme-linked technique thus provided a reliable discrimination between specific and non-specific (complementary or non-complementary to the probe used) PCR-amplified genomic DNA sequences. Intensities of signals obtained with the complementary PCR products (applied at the electrode at concentrations between 15 to 20 $\mu\text{g mL}^{-1}$ in 6- μL aliquots) were 20 to 30-times lower, compared to signals obtained for similar mass amounts of the target ODN (Fig. 3). This could be expected, considering relative contents of the specific DNA stretches forming duplexes with the probes in the tDNAs. While in the single-stranded 40-mer ODN, 50 % of the total DNA corresponded to the 20-nucleotide sequence recognized by the probe, proportions of the recognized stretches in the PCR amplicons were 3-4 %. Molar amounts of the specific sequences in the PCR amplicons per sample were, for the given DNA amounts, around 0.75 pmol. Such molar amounts corresponded to about 1 $\mu\text{g mL}^{-1}$ of the 40-mer **T^{p53}** ODN which gave a signal of comparable intensity (Fig. 3). Hence, these results suggest an excellent performance of the technique, providing well-defined and reliable responses not only for the model synthetic ODNs, but also for the “real” samples of the PCR-amplified genomic DNA elements.

2.2.1 Monitoring of gene expression using DNA hybridization

Gene expression is a process involving transcription of DNA sequence into RNA sequence, processing of the primary transcript into mRNA and translation of the mRNA sequence into amino acid sequence during proteosynthesis (Fig. 4A). The gene expression can in principle be monitored at the RNA or protein levels [1]. Specific mRNA can be detected, for example, using northern hybridization (total RNA isolated from biological material is separated by gel electrophoresis, blotted onto a membrane and hybridized with a gene-specific probe), or using an approach involving reverse transcription of RNA into cDNA, followed by PCR amplification of the cDNA using gene-specific primers (RT-PCR, Fig. 4A) [1, 32, 33]. RT-PCR has been widely applied in connection with various detection platforms,

including simple agarose gel electrophoresis of the amplified cDNA fragments (Fig. 4C), gene arrays employing cDNA hybridization with surface-confined capture probes, as well as real-time PCR techniques allowing precise qualitative analyses [34, 35].

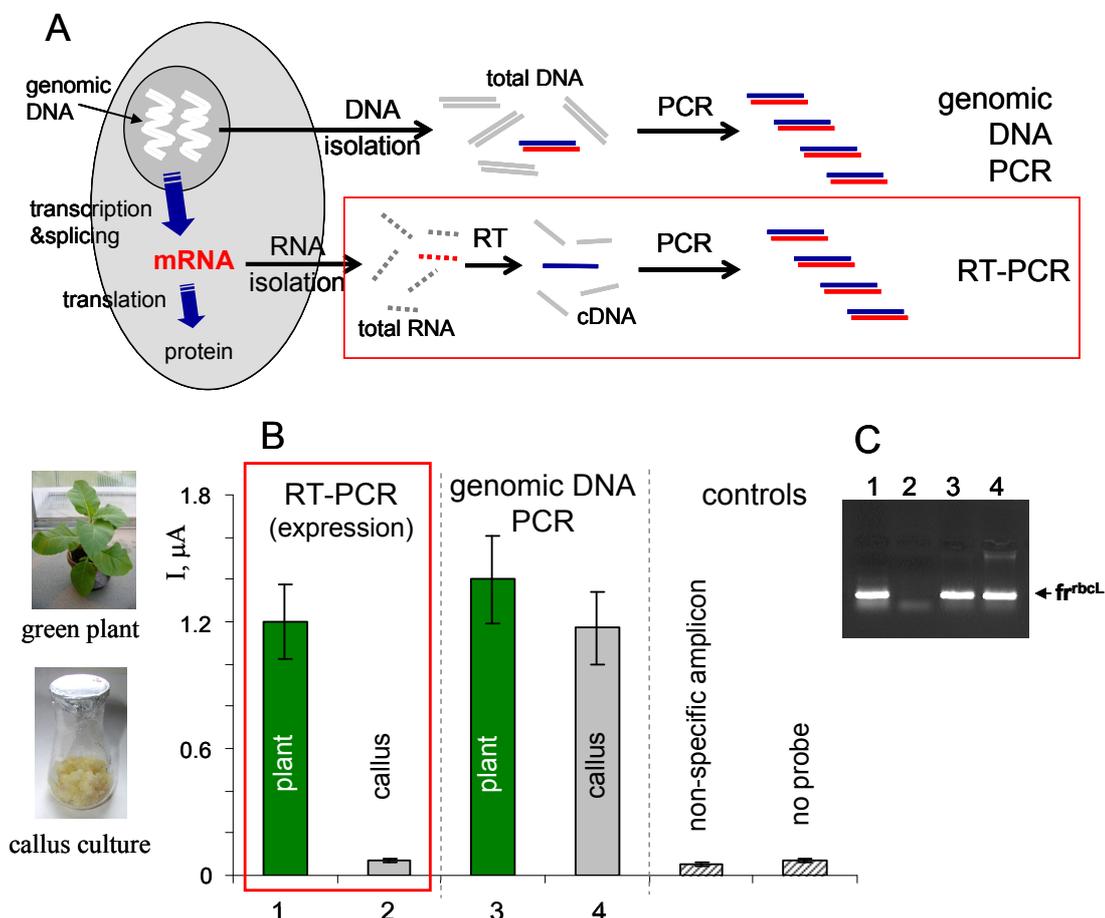


Figure 4. Monitoring of *rbcL* gene expression in plant tissues using the electrochemical enzyme-linked DNA hybridization assay. (A), scheme of the experiment. *RT-PCR*: total RNA isolated from tobacco tissues was reversely transcribed (RT) into cDNA using random primers, followed by PCR amplification of the *rbcL* gene fragment (fr^{rbcL}) using specific primers (see Table I). *Genomic DNA PCR*: the fr^{rbcL} fragment was amplified from total tobacco genomic DNA. (B), bar graph showing intensities of peak N obtained for the PCR products: (1-2), RT-PCR; (3-4), genomic DNA PCR; (1,3), green leaves; (2,4), non-green callus. The hybridization assays were performed as in Fig. 2B using biotinylated p^{rbcL} probe; *controls*: “non specific amplicon”, fr^{p53} was used as tDNA; “no probe”, fr^{rbcL} (resulting from RT-PCR of the green plant sample) was used as tDNA but no probe was subsequently added. (C), agarose gel electrophoresis of the PCR products 1-4 (the same numbering as in B).

Here we used the electrochemical enzyme-linked DNA hybridization technique in connection with RT-PCR for monitoring of expression of a gene *rbcL* [36] in two types of plant tissues, green leaves and non-green callus cultures of tobacco (*Nicotiana tabacum*). The *rbcL* gene, encoding one of

subunits of the enzyme Rubisco involved in photosynthesis, is known to be expressed in green parts of plants upon illumination, but not in the non-green plant cells [36]. Total RNA was isolated from the tobacco tissues and reversely transcribed into cDNA using random nonamer primers, and the fr^{rbcL} fragment was amplified using *rbcL*-specific primers (Table I). In parallel to this RT-PCR experiment, total genomic DNA was isolated from the plant material and used as a template for PCR amplification of the same fr^{rbcL} fragment. All PCR products were applied as tDNAs in the electrochemical enzyme-linked DNA hybridization assay. Positive signal observed in the RT-PCR experiment for green tobacco leaves was in agreement with the expected active *rbcL* expression in this tissue (Fig. 4B). On the contrary, response obtained for the *rbcL* non-expressing callus culture was at the level of negative controls. When the fr^{rbcL} fragment was amplified from the total genomic DNA (“genomic DNA PCR” in Fig. 4), positive signals were detected for both tobacco tissues, in agreement with presence of the gene in the plant genome regardless of the cell or tissue type (the sense of information gained from the latter experiment is “the gene is present”, while that obtained from the RT-PCR experiment means “the gene is active/inactive”). Control agarose gel electrophoresis (Fig. 4C) confirmed presence of the fr^{rbcL} amplicon in lanes corresponding to samples that gave positive responses in the electrochemical assay (Fig. 4B).

2.3 Primer extension-based DNA sensing

Sequence-specific DNA detection using various DNA labels need not necessarily involve DNA hybridization with labeled probes at surfaces. The markers, when available in the form of labeled dNTPs, can be introduced in specific DNA regions by DNA polymerases [16, 21-23, 25]. In this case, the sequence specificity is achieved through using a (unlabeled) probe which hybridizes with the tDNA of interest and serves as a primer for the labeled DNA synthesis (Fig. 5A). When the primer extension (PEX) reaction mixture contains a biotin-labeled dNTP (such as $\text{dU}^{\text{bio}}\text{TP}$), the biotin tags are introduced into the synthesized DNA stretch. Incorporation of multiple tags per PEX product (i.e., per a primer molecule) offers a possibility of signal enhancement [21]. The biotinylated PEX product can be detected using the electrochemical enzyme-linked assay depicted in Figure 1B. The procedure is analogous to that used above for the DNA hybridization assays (Fig. 1A) but incubation with the biotinylated probe is omitted.

Figure 2C shows responses obtained via the procedure shown in Fig. 1B for a biotinylated ODN (P^{p53}) or for $\text{dU}^{\text{bio}}\text{TP}$. While the ODN applied at the electrode in 0.4 μM solution yielded a well defined peak N, practically no signal was observed for $\text{dU}^{\text{bio}}\text{TP}$ concentration by 4 orders of magnitude higher (1 mM). This striking difference was in accord with different adsorbabilities of the two species at the electrode surface. As shown previously (reviewed in [37, 38]), monomeric nucleic acid components, in contrast to oligonucleotides, polynucleotides and natural nucleic acids, are not adsorbed at electrode surfaces strongly enough to resist medium exchange. This difference makes it possible to analyze (strongly adsorbing) nucleic acids in mixtures with (weakly adsorbing) bases, nucleosides or nucleotides by means of adsorptive transfer stripping (*ex situ*) voltammetry without any significant interference of the monomeric species. Here, the lack of signal of the $\text{dU}^{\text{bio}}\text{TP}$ made it in

principle possible to analyze PEX reaction mixtures using the electrochemical enzyme-linked assay without removal of the unreacted dNTPs.

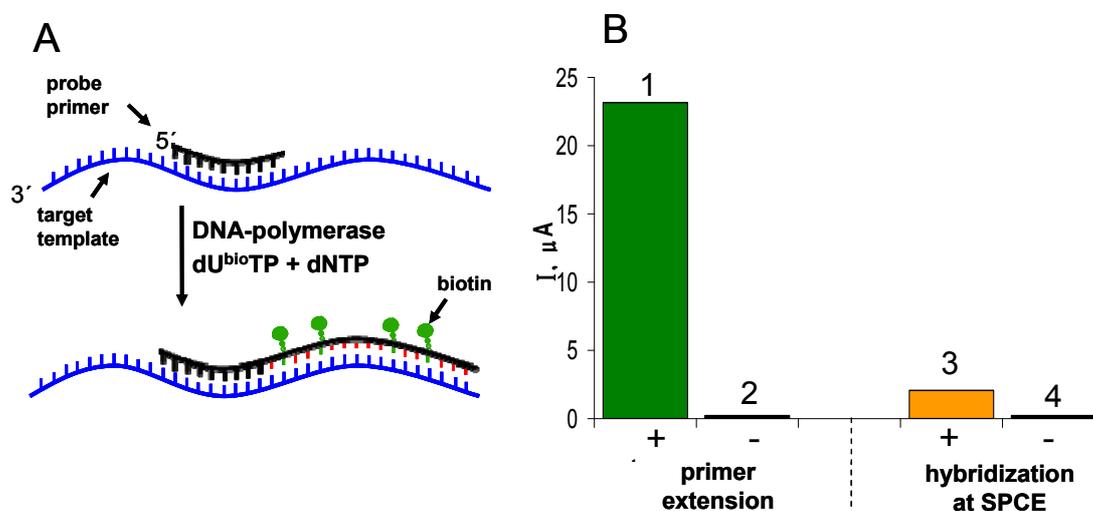


Figure 5. Primer extension-based electrochemical enzyme-linked DNA sensing. (A) Scheme of PEX incorporation of biotinylated nucleotides in DNA. A probe hybridizing with a complementary stretch in tDNA serves as a primer for DNA synthesis on the target template next to the primer binding site. When the dNTP mix contains a biotinylated dNTP (here, dU^{bio}TP), the biotin tags are introduced into the synthesized DNA strand. The PEX product is then adsorbed at the electrode and detected according to the scheme depicted in Fig. 1B. (B) Comparison of responses obtained for the fr^{p53} amplicon (1), via the PEX-based technique; or (3), via hybridization with the p^{p53} probe (as in Fig. 2B); (2), negative PEX control (no probe primer added to the fr^{p53} target template); (4), non-complementary p^{rbcl} probe was used in the DNA hybridization assay with the fr^{p53} tDNA.

2.3.1 PEX-based analysis of PCR products and monitoring of gene expression

The PEX-based technique was used to detect the fr^{p53} amplicon. The probe primer prim^{p53} was mixed with the PCR product, complete dNTP mix (involving dU^{bio}TP instead of dTTP) and a thermostable DNA polymerase. The mixture was subjected to a thermal cycle during which the PCR-amplified double-stranded DNA fragment was denatured, the probe primer hybridized with the template and the primer extension proceeded. The cycle was repeated ten times to enrich the sample for the biotin-labeled DNA. (It should be noted that the biotinylated dNTP can in principle be added to the PCR mixture, resulting in global modification of the amplicon molecules. Nevertheless, probing a specific sequence segment within the amplified DNA fragment (see Table I) is advantageous since it makes the PCR-based assays less prone to producing false positives due to erroneous amplification of non-specific products.) Finally, the PEX product was applied at the SPCE and the detection procedure was conducted according to Figure 1B. As shown in Figure 5B, an intense signal was detected for the primer prim^{p53} and target template fr^{p53} (involving a sequence complementary to the primer,

see Table I). Signal arising from the PEX experiment was by an order of magnitude more intense than that arising from hybridization of the fr^{p53} amplicon with the P^{p53} probe at the SPCE surface (while the amounts of the fr^{p53} fragments used per experiment were comparable, Fig. 5B). Enhancement of the signal intensity was in accord with incorporation of multiple biotin tags per probe primer hybridized with the target template; in the hybridization assay, only one biotin moiety per hybridization event was collected.

In next experiment, the RT-PCR products prepared for the tobacco tissues were analyzed by the PEX technique. Again, a strong signal was observed for *rbcL*-expressing green leaves (Fig. 6). For the non-green plant material, including the solid callus culture (Fig. 4) as well as TBY-2 cell suspension culture [39], only negligible background responses comparable to blank PEX mixture (with no target template added), were detected. Differentiation between the expressing and non-expressing tissues was in the PEX technique even better than in the hybridization assay (Fig. 4) due to specific enhancement of the positive signal.

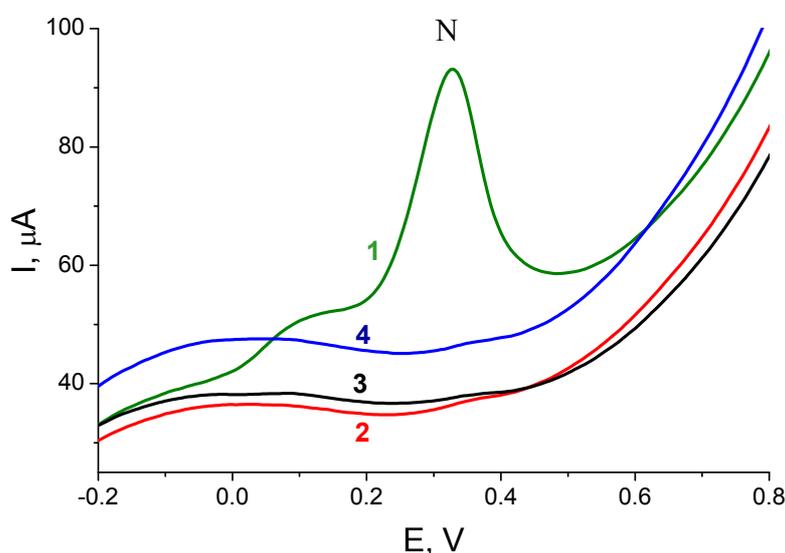


Figure 6. RT-PCR monitoring of gene expression in tobacco tissues using the PEX-based electrochemical enzyme-linked technique. Responses obtained for (1), green plant cells; (2) non-green solid callus culture; (3) non-green suspension cell culture TBY-2; and (4) blank PEX (no target template added to the PEX mixture). For more details, see Figs. 4 and 5.

2.4 Concluding remarks

We present DNA sensing techniques based on application of enzyme DNA labels and disposable screen-printed sensors. Target DNA adsorbed at the SPCE surface can be hybridized with probes bearing biotin tags for subsequent attachment of streptavidin-alkaline phosphatase conjugate. Owing to efficient SPCE surface blocking with BSA, non-specific adsorption of the biotinylated probes (and/or the SALP) is minimized, resulting in low background responses. Involvement of the soluble indicator is critical for the sensor performance because it can efficiently diffuse through the blocking BSA layer and produce the signal. Since the technique is “signal on” by its nature (no signal is produced in

absence of DNA hybridization but appears upon recognition of the target sequence by the labeled probe), presence of an excess of non-specific DNA or certain portion of unhybridized tDNA at the sensor surface does not affect the sensor responses. Comparative experiments using an alternative technique, based on a redox indicator methylene blue [27] (which is inherently sensitive to presence of sequences flanking the probe-recognized stretches in tDNAs), did not reveal any detectable differences between complementary and non-specific DNAs under the same conditions (not shown). An excellent discrimination between complementary and non-complementary nucleotide sequences of real PCR-amplified tDNAs can be achieved after short hybridization times (such as 60-120 s), making the technique applicable in rapid screening of series of biological samples.

In addition, we present for the first time an alternative approach involving incorporation of biotinylated nucleotides into DNA by primer extension. In this case, sequence specificity of the assay is not achieved via tDNA hybridization with labeled probe at the SPCE surface, but via recognition of the tDNA by a specific primer for the labeled DNA synthesis. We show that the incorporated biotin tags can be detected by the enzyme-linked electrochemical assay after adsorption of the PEX product at the SPCE (while unincorporated biotinylated dNTP does not produce the signal due to rather weak adsorption at the electrode). Owing to multiple biotin tags introduced per primer-tDNA hybrid in the PEX technique, a significant enhancement of the signal is achieved, compared to tDNA hybridization at the electrode surface with probes bearing one biotin per molecule. Both approaches have been successfully applied in monitoring of tissue-specific gene expression.

3. Experimental Section

3.1 Materials

Synthetic ODNs (see Table I) were purchased from VBC Biotech, random nonamers used for reverse transcription experiments from Sigma. Plasmid pT77 bearing wild type *p53* cDNA insert [40] (used as primary template for PCR amplification of the **fr**^{p53} fragment) was isolated from *E. coli* cells using Qiagen Plasmid Purification Kit and linearized with *Eco* RI restrictase (Takara). Total genomic DNA from the tobacco leaves, solid callus culture or the TBY-2 suspension culture [39] was isolated as described ([1] and references therein). Total RNA from the same plant material was isolated using RNeasy Plant Mini Kit (Qiagen), including treatment with RNase-free DNase I (DNase I Set, Qiagen). *Pfu* DNA polymerase and Streptavidin alkaline phosphate conjugate were obtained from Promega, DyNAzymeTM II DNA Polymerase from Finnzymes (Finland), reverse transcriptase SuperScript II from Invitrogen, unmodified nucleoside triphosphates (dATP, dTTP, dCTP and dGTP) from Sigma, Biotin-16-dUTP (dU^{bio}TP) from Roche, and 1-naphtyl phosphate disodium salt from Sigma. Other chemicals were of analytical grade.

Table 1. Nucleotide sequences of synthetic ODNs and PCR products used in this work.

Nucleotide sequence (5'→3')	acronym	note
CAGGCACAAACACGCACCTC(A) ₂₀	T ^{p53}	40-mer target ODN
GTTGTTGTTGTTGTTGTTGTT(A) ₂₀	NT ^{gtt7}	41-mer non-complementary ODN
GAGGTGCGTGTTTGTGCCTG	p ^{p53}	3'-biotinylated probe
TAGAAGATTCGGCAGCTACC	P ^{rbcL}	3'-biotinylated probe
TGCGTGTGGAGTATTTGGAT	prim ^{p53}	probe primer for PEX
TAGAAGATTCGGCAGCTACC	prim ^{rbcL}	probe primer for PEX
GAGGTTGTGAGGCGCTGCCC	p53-for	PCR primer
TCCTCTGTGCGCCGGTCTCT	p53-rev	PCR primer
GAGGTTGTGAGGCGCTGCCCCACCATGAGCGCTGCTCAGATAGCGATG GTCTGGCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATT TGCGTGT GGAGTATTTGGAT GACAGAAACACTTTTCGACATAGTGTGGTGGTGGCC TATGAGCCGCCTGAGGTTGGCTCTGACTGTACCACCATCCACTACA ACTA CATGTGTAACAGTTCCTGCATGGGCGGCATGAACCGGAGGCCCATCCTC ACCATCATCACACTGGAAGACTCCAGTGGTAATCTACTGGGACGGAACA GCTTT GAGGTGCGTGT TTGTGCCTGTCCTGGGAGAGACCGGCGCACAG AGGA	fr ^{p53}	PCR-amplified fragment of p53 cDNA (347-bp dsDNA) [±]
ATGTCACCACAAACAGAGAC	rbcL-for	PCR primer
CTCGATGCGGTAGCATCGCCCTTT	rbcL-rev	PCR primer
ATGTCACCACAAACAGAGACTAAAGCAAGTGTGGATTCAAAGCTGGTG TTAAAGAGTACAAATTGACTTATTATACTCCTGAGTACCAAACCAAGGAT ACTGATATATTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCACC TGAAGAAGCAGGGGCCG GGTAGCTGCCGAATCTTCTA CTGGTACATG GACAACTGTATGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGG CGATGCTACCGCATCGAG	fr ^{rbcL}	PCR-amplified fragment of rbcL cDNA (264-bp dsDNA) [±]

[±]The PCR-amplified genomic DNA fragments are double-stranded; only the forward strands are shown. In **fr**^{p53}, sequence corresponding to the biotinylated **P**^{p53} probe (used in hybridization experiments) is **bold**, that corresponding to the probe primer **prim**^{p53} (used in PEX experiments) is **bold underlined** (both probes are derived from the forward strand and hybridize with the reverse strand). In **fr**^{rbcL}, the biotinylated probe **P**^{rbcL} and the probe primer **prim**^{rbcL} are derived from the same stretch in the reverse strand and bind to the **bold underlined** site in the forward strand.

3.2 PCR and RT-PCR

Amplification of the **fr^{p53}** fragment: 500 ng of the pT77 template was mixed with p53-for and p53-rev primers (0.5 μM each), *Pfu* DNA polymerase (3 U) and mix of standard dNTPs (125 μM each) in a total volume of 100 μl . The PCR involved 30 cycles (denaturation 94 °C/90 s, annealing 60 °C/120 s, polymerization 72 °C/180 s). The same procedure, using rbcL-for and rbcL-rev primers, was used for amplification of the **fr^{rbcL}** fragment from the total tobacco genomic DNA.

RT-PCR: Reverse transcription of the total tobacco RNA was performed according to manufacturer's instructions. PCR amplification of the *rbcL* reverse transcript was conducted as above using the rbcL-for and rbcL-rev primers.

For the DNA hybridization and PEX experiments, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen).

3.3 Primer extension

0.7 μM primer (**prim^{p53}** or **prim^{rbcL}**) was mixed with 300 ng of the respective template, 1 U of DyNAzyme II DNA polymerase, and a mix of dATP, dCTP, dGTP and dU^{bio}TP (125 μM each) in a total volume of 30 μl . The reaction was conducted in 10 thermal cycles (94 °C/90 s, 60 °C/120 s, 72 °C/180 s).

3.4 Preparation of the screen-printed carbon electrodes

The SPCEs were prepared by coating of the carbon ink (Gwent C 10903P14, UK) through a stencil, using squeegee of the printing device (SP-200, MPM), onto an inert laser pre-etched ceramic support (Coors Ceramic). The resulting plates were dried at 60 °C for 1h. Active surface area of the SPCE sensors was delimited by coating the adjacent part of the SPCE stripe by nail-varnish.

3.5 Electrochemical enzyme-linked assays

3.5.1 Hybridization at the SPCE surface

Target DNAs were adsorbed at the active SPCE surface from 6- μL drops of solutions containing either 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5 (buffer H; used for adsorption of synthetic ODNs) or 20 mM NaOH + 180 mM NaCl (used for the PCR-amplified DNA fragments). Accumulation time was always 120 s. Unoccupied SPCE surface was then blocked by incubation of the electrode in stirred solution of 2 % bovine serum albumin (BSA) in PBS (0.28 M NaCl, 5.5 mM KCl, 24 mM NaHPO₄, 3.5 mM KH₂PO₄, pH 7.4) for 120 s. After rinsing by PBS, 6 μL of the probe solution (5 $\mu\text{g mL}^{-1}$) in buffer H was applied at the electrode surface for 120 s. Then the electrode was rinsed by PBS and 6- μL of SALP solution (100-times diluted stock in PBS containing 2 % BSA) was applied at the SPCE for 120 s. The modified electrode was then washed in PBS containing 0.05 % of Tween 20 for 30 s and then in PBS for another 30 s. Finally, the electrode was placed into voltammetric cell containing 3 mL of background electrolyte (0.5 M K₂CO₃ and 0.5 M NaHCO₃, pH 9.5) containing 5 mM 1-naphtyl phosphate. Enzymatically produced 1-naphtol was detected after a short incubation period using the voltammetric peak N (Fig. 2). Each experiment involved two voltammetric scans with the same sensor.

The first was recorded after 20 s of the electrode incubation in the substrate solution and the other, recorded after another 60 s, was evaluated as the analytical signal [13].

3.5.2 Analysis of biotinylated PEX products

The experiments were carried out as above but hybridization with the biotinylated probe was omitted. Briefly, the biotinylated DNA was adsorbed at the SPCE, followed by surface blocking by BSA, binding of SALP and production of the 1-naphthol indicator (see Fig. 1 for schemes of the procedures).

3.6 Voltammetric measurements

All measurements were performed with a CHI440 Electrochemical Workstation (CH Instruments, Inc., USA) connected to a three-electrode system (with the SPCE as working, Ag/AgCl/3M KCl as reference and platinum wire as counter electrode). The electroactive indicator 1-naphthol was detected using linear sweep voltammetry (LSV) in 0.5 M K₂CO₃ and 0.5 M NaHCO₃, pH 9.5, with initial potential -0.5 V, end potential +0.9 V, scan rate 1 V s⁻¹, potential step 5 mV.

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