DNA Labeling

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Aminophenyl- and Nitrophenyl-Labeled Nucleoside Triphosphates: Synthesis, Enzymatic Incorporation, and Electrochemical Detection**

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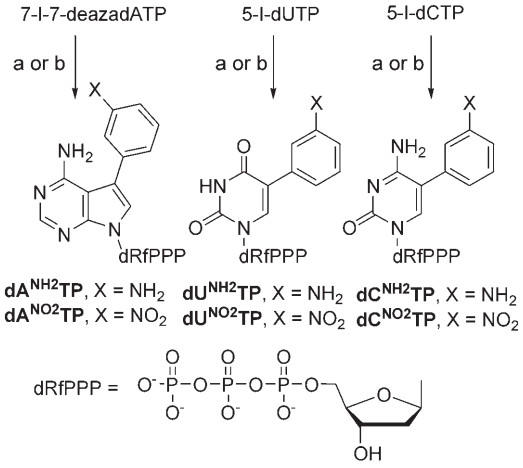
DNA biosensors and chips are broadly utilized[1] in the life sciences. Electrochemical detection[2] is a less expensive but comparatively sensitive alternative to common optical methods. Although nucleic acids are electroactive themselves, diverse electroactive tags are used to increase sensitivity and specificity.[2] Besides widely used DNA tags based on metal complexes,[3] quantum dots,[4] or phenothiazine dyes,[5] some simple organic derivatives (such as aromatic amines or nitro compounds) exhibit distinct electrochemical activity,[6] thus making them candidates for nucleic acid labeling. In particular, the nitro group appears promising for sensitive detection because of the high number of electrons (four or six) collected per nitro group reduction.[6] So far, neither amino nor nitro groups have been used as specific electroactive DNA markers.

Some modified 2′-deoxyribonucleoside triphosphates (dNTPs) bearing substituents at the nucleobase can be enzymatically incorporated into DNA by polymerases. This approach has been used[7] for the construction of functionalized nucleic acids bearing diverse functional groups. Recently, aqueous-phase cross-coupling reactions of unprotected halogenated nucleoside triphosphates with boronic acids[8] or acetylenes[9] were developed and used in combination with polymerase incorporation for the two-step construction of modified nucleic acids,[9] including ferrocene-labeled oligonucleotides (ONs).[10]

Herein, we report the synthesis of nucleoside triphosphates bearing aminophenyl and nitrophenyl groups attached to a nucleobase, their enzymatic incorporation, and the preliminary electrochemical properties of the labeled ONs. We expected that the fully conjugated aromatic system would respond well to the electronic changes arising from incorporation into nucleic acids, and result in changes of the redox potential of the label.

The modified dNTPs were prepared by the single-step aqueous-phase cross-coupling reactions of halogenated dNTPs, in analogy to our previously developed procedures.[8,9] The Suzuki–Miyaura reaction of 7-iodo-7-deaza-2′-deoxyadenosine 5′-triphosphate (7-I-7-deaza-dATP), 5-iodo-2′-deoxyuridine 5′-triphosphate (5-I-dUTP), or 5-iodo-2′-deoxycytidine 5′-triphosphate (5-I-dCTP) with either 3-aminophenyl- or 3-nitrophenylboronic acid (Scheme 1) gave the



Scheme 1. Reagents and conditions: 3-aminophenylboronic acid (a) or 3-nitrophenylboronic acid (b) and Cs_2CO_3, $Pd(OAc)_2$, $P(C_6H_4\text{-}3\text{-}SO_3Na)_3$, H_2O/CH_3CN (2:1).

corresponding 3-aminophenyl or 3-nitrophenyl derivatives of dATP (**dA^{NH_2}TP**, **dA^{NO_2}TP**), dUTP (**dU^{NH_2}TP**, **dU^{NO_2}TP**), or dCTP (**dC^{NH_2}TP**, **dC^{NO_2}TP**) in acceptable yields after isolation by reversed-phase HPLC (40–43% for NH_2, 26–28% for NO_2). This straightforward and efficient approach gives directly the desired functionalized dNTPs, which are suitable as substrates for polymerase incorporation.

All conjugates were successfully incorporated into ONs through primer extension (PEX) catalyzed by either the Klenow (exo-) DNA polymerase fragment or a thermostable DyNAzyme DNA polymerase (Table 1). Incorporation of the labeled nucleotides into different nucleotide sequences was tested, including those accommodating the conjugates at separated positions, at positions alternating with another base

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Table 1: Primer and templates used for PEX experiments.[a]

primer	5′-CATGGGCGGCATGGG-3′
temp^{AT}	5′-**TATATATATAT**CCCATGCCGCCCATG-3′
temp^{AC}	5′-**TGTGTGTGTGT**CCCATGCCGCCCATG-3′
temp^{TC}	5′-**AGAGAGAGAGA**CCCATGCCGCCCATG-3′
temp^{C}	5′-**CCCG**CCCATGCCGCCCATG-3′
temp^{T}	5′-**CCCA**CCCATGCCGCCCATG-3′
temp^{A}	5′-**CCCT**CCCATGCCGCCCATG-3′
temp^{rnd16}	5′-**CTAGCATGAGCTCAGT**CCCATGCCGCCCATG-3′

[a] Templates used in experiments involving the magnetoseparation procedure (see the Supporting Information) were biotinylated at the 5′ end; analogous acronyms are used for the corresponding PEX products.

(doublet repeats), or within homonucleotide blocks. Generally, synthesis of DNA stretches with the conjugates incorporated between two unmodified bases proceeded well along the whole template (Figure 1). Incorporation of $\mathbf{A}^{\mathbf{NH_2}}$, $\mathbf{A}^{\mathbf{NO_2}}$,

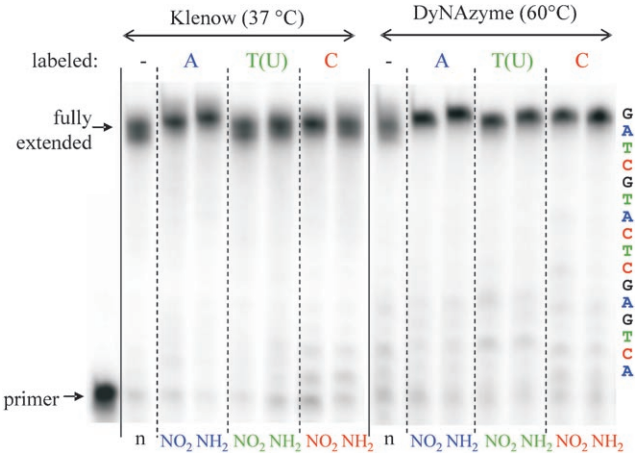

Figure 1. Denaturing PAGE analysis of PEX products synthesized on template temp^{rnd16} using dNTP mixes containing one of the nitrophenyl or aminophenyl dNTP conjugates (**dA^{NH_2}TP**, **dA^{NO_2}TP**, **dU^{NH_2}TP**, **dU^{NO_2}TP**, **dC^{NH_2}TP**, or **dC^{NO_2}TP**) complemented by the remaining three unmodified dNTPs.

$\mathbf{U}^{\mathbf{NH_2}}$, and/or $\mathbf{U}^{\mathbf{NO_2}}$ at mutually adjacent positions was also easy (in contrast to incorporation of ferrocene-labeled U or A into homonucleotide blocks[10]). Incorporation of $\mathbf{C}^{\mathbf{NH_2}}$ or $\mathbf{C}^{\mathbf{NO_2}}$ next to any other conjugate was less feasible, and resulted in early termination of PEX, especially when DyNAzyme was used (see the Supporting Information for details). A systematic study of incorporation of the aminophenyl- or nitrophenyl-labeled nucleobases into various DNA sequences (by PEX or PCR) is in progress.

All labeled dNTPs produced well-defined voltammetric signals as a result of the reduction of the nitro group (Figure 2a) or oxidation of the amino group (Figure 2b). The peak arising from irreversible reduction of the nitro group appeared at potentials ranging from −615 to −665 mV, depending on the nucleobase to which the nitrophenyl moiety was coupled (Table 2). Similarly, the potentials of irreversible oxidation of the amino group varied between +630 and +685 mV. These results confirmed our assumption that the

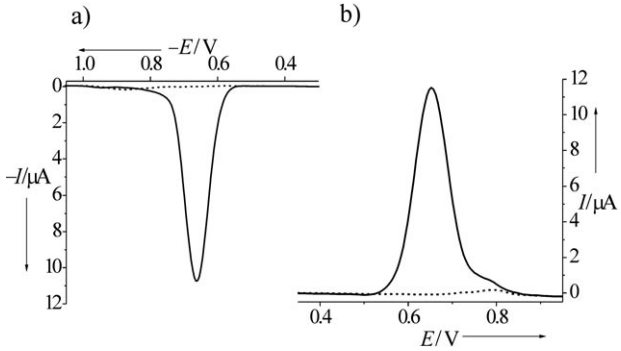

Figure 2. Square-wave voltammetry (SWV) signals resulting from a) nitro group reduction in **dU^{NO_2}TP** or b) amino group oxidation in **dU^{NH_2}TP** at a pyrolytic graphite electrode. Dotted curves correspond to unmodified dTTP. For more details, see the Supporting Information.

Table 2: Apparent redox potentials of nitrophenyl or aminophenyl labels in dNTPs or PEX-incorporated nucleotides in ONs.

N	dN^{NO_2}TP	pex(NO$_2$)[b]	dN^{NH_2}TP	pex(NH$_2$)[b]
C	−615[a]	−650 ± 5	+685	+810 ± 5
A	−635	−665 ± 5	+630	+760 ± 5
U	−665	−685 ± 5	+650	+760 ± 5

[a] Potentials of SWV peaks (mV against Ag/AgCl/3 M KCl reference electrode). [b] Average of values obtained for pex^{C}, pex^{T}, pex^{A}, pex^{TC}, pex^{AC}, pex^{AT}, and pex^{rnd16}, each containing only one type of labeled nucleotide.

electronic changes on the nucleobase reflect the changes in redox potential of the label resulting from the conjugate aromatic system.

Single-stranded ONs bearing the labeled nucleobases incorporated by PEX were isolated by the magnetoseparation technique described previously[10,11] and analyzed by ex situ SWV. The resulting voltammograms revealed the presence of the nitro or amino labels (depending on the kind of labeled dNTP included in the reaction mixture; Figure 3). As a result

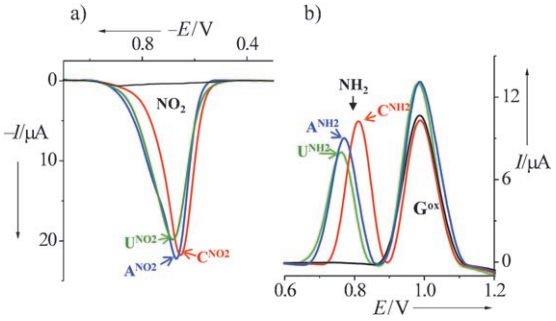

Figure 3. SWV responses of pex^{rnd16} products bearing a) nitrophenyl or b) aminophenyl labels synthesized using mixtures of labeled dNTPs (blue, **dA^{NO_2}TP** or **dA^{NH_2}TP**; green, **dU^{NO_2}TP** or **dU^{NH_2}TP**; red, **dC^{NO_2}TP** or **dC^{NH_2}TP**) with unlabeled dNTPs to complete the synthesized sequence (see Figure 1), and 5′-biotinylated templates. Black curves correspond to the unlabeled PEX product. The PEX reaction was catalyzed by Klenow polymerase. Prior to SWV measurements, the extended primer strands were isolated by a magnetoseparation procedure (see the Supporting Information). G^{ox}: signal from the electrooxidation of guanine.

of the conjugate aromatic system, the apparent redox potentials of the amino or nitro labels responded to incorporation into ONs. Compared to the respective dNTP conjugates, reduction signals from ON-incorporated nitro labels were shifted to more negative potentials and the oxidation signals of the amino labels to more positive potentials (Table 2). Although we do not have an explanation for this dichotomy at this stage, the resulting changes in redox potential are reproducible and analytically useful.

In principle, the irreversible electrochemistry of the nitro and amino groups hampers utilization of ONs bearing these tags in some specific detection systems based on reusable redox-labeled recognition layers (for example, electrochemical molecular beacons[12]). Nevertheless, practical bioanalytical applications of modified nucleic acids do not necessarily require regeneration of the labeled nucleic acid molecules. Other aspects, such as the possibility of parallel detection of more labels, are often more important. Differences in electrochemical processes that give rise to specific signals produced by either nitro or amino DNA tags offer perfect discrimination between the two labels (one label is irreversibly reduced while the other is irreversibly oxidized, thus their signals cannot overlap and cannot be mistaken). Hence, both types of markers incorporated in the same DNA (ON) molecule can be readily detected (see the Supporting Information for details).

Typical assays based on sequence-specific incorporation of labeled dNTPs involve DNA "minisequencing" focused on detection of point mutations or single-nucleotide polymorphisms (SNPs).[13] Figure 4 shows examples of PEX-based

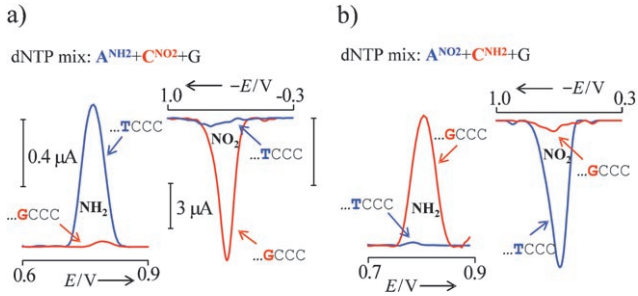

Figure 4. PEX probing of a SNP using nitro- and amino-labeled nucleotides. The PEX reactions were conducted with templates temp^A or temp^C (sequences complementary to the synthesized stretches shown) and a mixture of either a) **dA^{NO_2}TP + dC^{NH_2}TP** or b) **dA^{NH_2}TP + dC^{NO_2}TP** (complemented with unlabeled dGTP). Sections of voltammograms spanning the incorporated label signals are shown. Other details as in Figure 2.

probing of T/G SNPs in model ON templates temp^A and temp^C (Table 1). The dNTP mix used for the PEX contained labeled dATP or dCTP, one always bearing a nitro tag and the other an amino tag. For the temp^A and **dA^{NH_2}TP + dC^{NO_2}TP** mix, a well-defined NH_2 peak but not NO_2 peak was observed, in agreement with specific incorporation of **A^{NH_2}** (Figure 4a). For temp^C and the same dNTP mixture, the NO_2 peak was specifically detected, thus indicating the presence of G (instead of T) in the template. Inverse responses were detected when **dA^{NO_2}TP + dC^{NH_2}TP** were added to the reaction mixture (Figure 4b). For temp^T and dNTP mixes involving labeled dUTP, the results were analogous. Taken together, these data illustrate the applicability of the dNTP conjugates in reliable SNP minisequencing through electrochemical detection of a single nitro- or amino-labeled nucleobase specifically incorporated in the probe ON.

Other bioanalytical applications can involve, for example, sandwich hybridization assays with nitro or amino end-labeled reporter probes (RPs). Incorporation of multiple modified bases in the RP single-stranded tail can provide amplification of the electrochemical signal, and probes bearing diverse tags can be utilized for "multicolor" electrochemical DNA sensing.[2,3] Research focused on other analytical applications of nitro- or amino-modified nucleic acids is now in progress.

In conclusion, we have developed a facile single-step synthesis of aminophenyl- and nitrophenyl-containing dNTPs through cross-coupling reactions. The modified dNTPs were efficiently incorporated by DNA polymerases to form NH_2- or NO_2-modified ONs. Both types of modifications serve as excellent electrochemical labels detectable by either oxidation (NH_2) or reduction (NO_2), which allows perfect discrimination between the two tags incorporated in the same DNA molecule. In addition, the redox potentials of both labels differ depending on the nucleobase and respond to incorporation into ONs, which could be analytically useful.

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