DOI: 10.1002/chem.200701249

Ferrocenylethynyl Derivatives of Nucleoside Triphosphates: Synthesis, Incorporation, Electrochemistry, and Bioanalytical Applications

Petra Brázdilová,[a, c] Milan Vrábel,[b] Radek Pohl,[b] Hana Pivoňková,[a] Luděk Havran,[a] Michal Hocek,*[b] and Miroslav Fojta*[a]

Abstract: Modified dATP (2′-deoxyadenosine-5′-triphosphate) and dUTP (2′-deoxyuridine-5′-triphosphate) bearing ferrocene (Fc) labels linked via a conjugate acetylene spacer were prepared by single-step aqueous-phase cross-coupling reactions of 7-iodo-7-deaza-dATP or 5-iodo-dUTP with ethynylferrocene. The Fc-labeled dNTPs were good substrates for DNA polymerases and were efficiently incorporated to DNA by primer extension (PEX). Electrochemical analysis of the 2′-deoxyribonucleoside triphosphates (dNTPs) and PEX products revealed significant differences in redox potentials of the Fc label bound either to U or to 7-deazaA and between isolated dNTPs and conjugates incorporated to DNA. Prospective bioanalytical applications are outlined.

Keywords: cross-coupling · DNA · electrochemistry · ferrocene · nucleotides · polymerase

Introduction

DNA biosensors and chips[1] are increasingly used in current molecular biology, biochemistry, and biomedicine to analyze nucleotide sequences of DNAs or RNAs, search for mutations, monitor gene expression, and so on. In addition to optical methods, electrochemical detection has attracted attention as a less expensive alternative offering comparable sensitivity.[2] Ferrocene[3] (Fc) is a privileged label for electrochemical detection of biomolecules. In nucleic acids,[4,5,6] it has been attached to sugar or nucleobase parts via diverse linkers and used for several applications including detection of single-point mutations. An Fc marker attached directly to a nucleobase via conjugate linker (e.g., acetylene) would be an attractive tool for further applications due to electronic conjugation of the whole system. Attempted incorporation of 5-Fc-ethynyl-2′-deoxyuridine caused cyclization to the furopyrimidine derivative,[5] whereas attempts at synthesis of analogous Fc-ethynyl derivatives of purines were unsuccessful[7,8] due to the limited stability of Fc in the phosphoramidite synthesis of oligonucleotides (ONs).

Modified 2′-deoxyribonucleoside triphosphates (dNTPs) bearing substituents at the nucleobase are often good substrates of DNA polymerases and can be used for construction of functionalized nucleic acids bearing diverse functional groups,[9] including Fc[6] attached through a nonconjugate linker, by enzymatic incorporation (by primer extension or PCR). Recently, aqueous-phase cross-coupling reactions of unprotected halogenated nucleoside triphosphates with boronic acids[10,11] or acetylenes[12] were developed and used in combination with polymerase incorporation for a two-step construction of modified nucleic acids. This approach might be a good alternative for the construction of ON probes bearing conjugated Fc markers on nucleobases, in which the difficulties with chemical synthesis of ONs are circumvented. Here we report on the synthesis of ferrocenylethynyl derivatives of nucleoside triphosphates, their incorporation, and preliminary electrochemical properties of the ONs.

[a] P. Brázdilová, Dr. H. Pivoňková, Dr. L. Havran, Prof. Dr. M. Fojta
Institute of Biophysics, v.v.i.
Academy of Sciences of the Czech Republic
Kralovopolska 135, 61265 Brno (Czech Republic)
Fax: (+420) 541-211-293
Homepage: http://www.ibp.cz/labs/LBCMO/
E-mail: fojta@ibp.cz

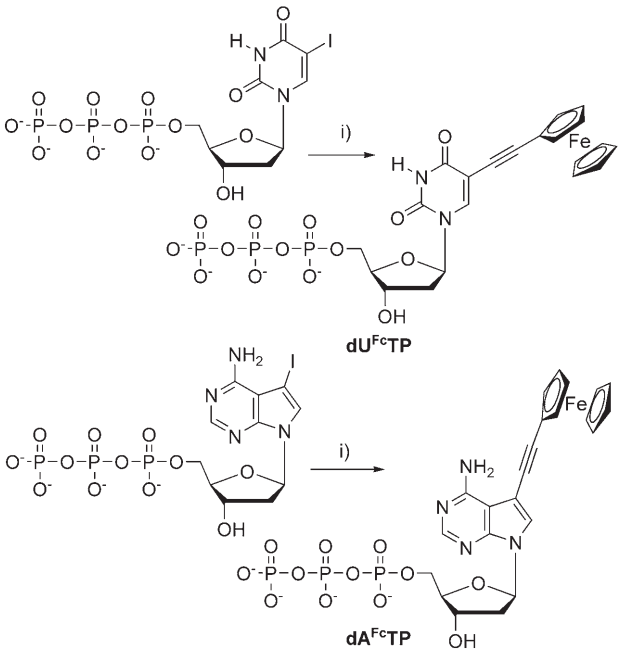
[b] M. Vrábel, Dr. R. Pohl, Prof. Dr. M. Hocek
Institute of Organic Chemistry and Biochemistry, v.v.i.
Academy of Sciences of the Czech Republic
Gilead Sciences & IOCB Research Center
Flemingovo nam. 2, 16610 Prague 6 (Czech Republic)
Fax: (+420) 220-183-559
Homepage: http://www.uochb.cas.cz/hocekgroup
E-mail: hocek@uochb.cas.cz

[c] P. Brázdilová
Department of Analytical Chemistry
Faculty of Chemical Technology
University of Pardubice
nam. Cs. legii 565, CZ-532 10 Pardubice (Czech Republic)

Supporting information for this article is available on the WWW under http://www.chemeurj.org or from the author.

Results and Discussion

Synthesis of Fc-modified dNTPs: Fc-modified dNTPs were synthesized by the direct single-step aqueous-phase cross-coupling reactions of halogenated dNTPs in analogy to our previously developed procedures.[10,11] Based on our previous experience that 8-substituted dATPs are not efficiently incorporated,[11] we have chosen 7-substituted 7-deaza-adenine as surrogate for the adenine base. Thus, the Sonogashira reaction of 5-iodo-2′-deoxyuridine 5′-triphosphate (5-I-dUTP) or 7-iodo-7-deaza-2′-deoxyadenosine (7-I-7-deaza-dATP) with ethynylferrocene proceeded in the presence of $Pd(OAc)_2$, $P(C_6H_4\text{-}3\text{-}SO_3Na)_3$ (TPPTS), CuI, and Et_3N in water/acetonitrile (2:1) at 65 °C for one hour (Scheme 1) to give the corresponding ferrocenylethynyl derivatives of dUTP ($dU^{Fc}TP$) or dATP ($dA^{Fc}TP$) in acceptable yields of 42 and 48 %, respectively, after isolation by reverse-phase HPLC (RP HPLC). This approach is very straightforward and efficient despite the moderate yields and allows an expeditious one-step (protection-free) synthesis of the desired Fc-modified dNTPs suitable as substrates for polymerase incorporations.



Scheme 1. Synthesis of Fc-modified dNTPs. Conditions: i) Ethynylferrocene, $Pd(OAc)_2$, TPPTS, CuI, Et_3N, acetonitrile/H_2O 1:2 (yields: 42 % $dU^{Fc}TP$; 48 % $dA^{Fc}TP$).

Incorporation of Fc-labeled nucleotides into oligonucleotides: This was performed by means of primer extension (PEX) catalyzed by either a Klenow (exo-) DNA polymerase fragment, or a thermostable DyNAzymeII DNA polymerase. Different ON templates were tested, including random-sequence stretches containing all four DNA bases or only dA•dT (dA•dU) pairs, as well as doublet repeats accommodating the Fc conjugates at positions alternating with another base in RR, RY/YR, or YY steps. Generally, polyacrylamide gel electrophoretic (PAGE) analysis of the PEX products showed that both U^{Fc} or A^{Fc} could be incorporated at separated positions (between unmodified neighboring bases) in all nucleotide sequences tested. On the other hand, incorporation of the conjugates in adjacent positions was less feasible, causing the PEX reaction to stop at such sites. Reactions catalyzed by the Klenow (exo-) enzyme (at 37 °C, in contrast to the DyNAzyme-catalyzed reactions performed at 60 °C) displayed higher frequency of erroneous nucleotide incorporation (poorer fidelity of the template reading) specifically when only a $dN^{Fc}TP$ was present in the PEX mixture (Figure 1). Such behavior was in agreement with less stringent conditions (lower temperature) and absence of the proofreading 3′→5′ exonuclease activity in this enzyme. With DyNAzyme, the tendency to PEX early termination at sites of clustered conjugate incorporation was more pronounced (Figure 1; for more examples see the Supporting Information). From these observations it can be tentatively concluded that DyNAzyme (or another thermostable polymerase) may be the enzyme of choice in applications requiring precise incorporation of the labeled nucleotides (such as detection of polymorphisms, searching for the homonucleotide blocks and so on). The fact that the

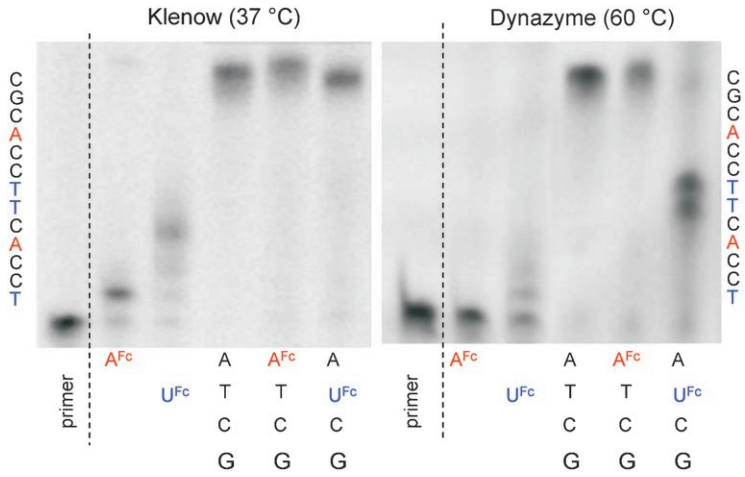
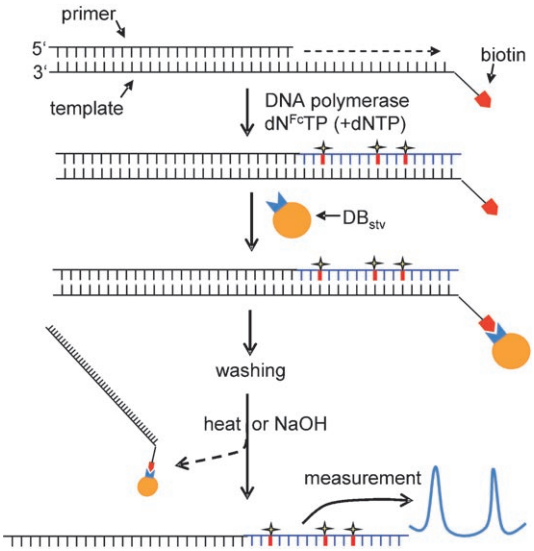


Figure 1. Denaturing PAGE analysis of PEX products synthesized on a $temp^{2A}$ template by using $dA^{Fc}TP$ and/or $dU^{Fc}TP$ (complemented with unmodified dNTPs). Nucleotide sequence of the synthesized stretch is shown along the panel edges; composition of the dNTP mixes at the bottom. PEX reactions in the left panel were performed with Klenow (exo-) polymerase at 37 °C, those in the right panel with a thermostable DyNAzyme polymerase at 60 °C.

Klenow enzyme is less prone to early termination of the PEX reaction (Figure 1) apparently renders it to be more convenient for incorporation of a large number of the Fc tags into the synthesized DNA strand (to achieve signal amplification, see below). A systematic study of Fc conjugate incorporation into various DNA sequences (by PEX or PCR) is under way and results will be published elsewhere.

To make the Fc-labeled strands of the PEX products ready for the electrochemical analysis, a simple magnetoseparation protocol was used (Scheme 2). Similar techniques



Scheme 2. PEX incorporation of nucleotide-Fc conjugates and separation of the labeled strand by using biotinylated templates and streptavidin-coated magnetic beads (DB_{stv}).

have recently been proposed to improve electrochemical detection of DNA hybridization or DNA protein interactions.[13] Briefly, the PEX reaction was performed with a 5′-biotinylated template. The double-stranded product was captured at streptavidin-coated magnetic beads (DB_{stv}), followed by separation from components of the reaction mixture (unconsumed dNTPs, proteins, or detergents that might cause interferences in the voltammetric measurements) by repeated magnetoseparation and re-suspension of the beads in the washing medium. The extended primer strand was released from the DB_{stv} via thermal or alkaline denaturation of the duplex followed by the ex situ electrochemical analysis.

UV spectra of the $dU^{Fc}TP$ conjugate after exposure to conditions of PEX or the magnetoseparation procedure did not show any significant absorption at 350 nm which proved that neither of these treatments caused the cyclization of the ethynyl-bridged U^{Fc} conjugate to furopyrimidine.[5] Thus, unlike the unsuccessful solid-phase phosphoramidite ON synthesis,[5] the PEX incorporation proceeds efficiently without side reactions (see the Supporting Information for more details).

Electrochemical analysis of the ferrocenylethynyl dNTP conjugates and labeled PEX products: Square-wave voltammetric (SWV) responses of $dU^{Fc}TP$ and $dA^{Fc}TP$ at a pyrolytic graphite electrode (PGE) are compared with the response of uncoupled Fc in Figure 2A. The free Fc yielded a

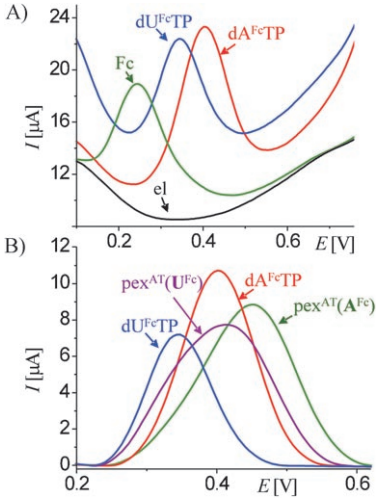


Figure 2. A) Sections of in situ square-wave voltammograms showing reversible redox signal of free Fc (green), $dU^{Fc}TP$ (blue), and $dA^{Fc}TP$ (red) (all substances 10 μM). B) Sections of baseline-subtracted voltammograms displaying the Fc tag signals measured for $dU^{Fc}TP$ (blue), $dA^{Fc}TP$ (red), and PEX products $pex^{AT}(U^{Fc})$ (magenta) and $pex^{AT}(A^{Fc})$ (green). The PEX products were prepared by using Klenow (exo-) DNA polymerase on the $temp^{AT}$-bio template, purified by the magnetic DB_{stv} technique (Scheme 2) and measured by using the ex situ SWV.

well-developed peak due to a reversible one-electron Fe^{II}/Fe^{III} redox process at 0.245 V. Similar reversible signals were observed with both $dU^{Fc}TP$ and $dA^{Fc}TP$, but their potentials exhibited remarkable positive shifts (by 105 and 155 mV, respectively), compared to the uncoupled Fc. Such behavior was in agreement with previously published data[7,14] showing positive shifts of the redox potential of Fc[7] or ruthenium complexes[14] coupled to nucleobase moieties via conjugate linkers. Electron-withdrawing effects of the electronically conjugate aromatic nucleobases make oxidation of the metal complexes more difficult, compared to uncoupled complexes, resulting in the positive shifts of the apparent redox potentials.[7,14] Effect of the 7-deaza-adenine on redox potential of the Fc tag was more significant than that of the uracil moiety (Figure 2). The resulting difference in the peak potentials (about 50 mV) was sufficient to enable differentiation between the conjugates.

Figure 3 shows ex situ SWV response of a $pex^{AT}(U^{Fc})$ product synthesized on a $T(AT)_6$ template ($temp^{AT}$) by using a mix of $dU^{Fc}TP$ and unlabeled dATP. The peak U^{Fc} around 0.415 V corresponds to the introduced Fc tags, whereas the peak G^{ox} at 0.970 V corresponds to oxidation of guanine residues in the ON (the primer contains 8 guanines, see Figure 3). The peak potential of incorporated U^{Fc} was 75 mV more positive than the corresponding peak of $dU^{Fc}TP$ (Figure 2B, Table 1). When (unlabeled) dTTP was

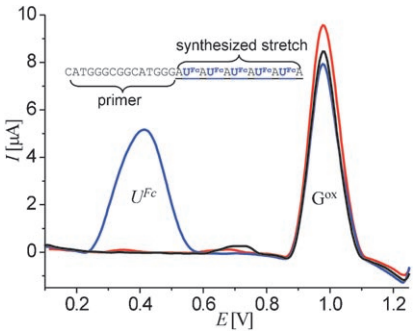


Figure 3. Ex situ SWV responses of pex^{AT}(U^{Fc}) (blue). The PEX product was prepared by using dU^{Fc}TP combined with unlabeled dATP; other details as in Figure 1. Controls: Unlabeled pex^{AT} product (mix of unlabeled dTTP+dATP was used; red); negative PEX reaction (dU^{Fc}TP+dATP without DNA polymerase, black). Inset: nucleotide sequence of the pex^{AT}(U^{Fc}) product.

Table 1. Apparent redox potentials of A^{Fc} or U^{Fc} in dNTPs or PEX products.

	A^{Fc}	U^{Fc}
dN^{Fc}TP	0.400[a]	0.350
pex^{AT} (ATATATATATA)	0.450	0.415
pex^{ATnrI} (ATATTTATATT)	0.435	0.395
pex^{AG} (AGAGAGAGAG)	0.430	–
pex^{1A} (TCCTCTTACTCGC)	0.440	–
pex^{2A} (TCCACTTCCACGC)	0.440	–

[a] SWV peak potentials measured against Ag/AgCl/3 M KCl reference electrode. Sequences of the synthesized ON stretches are shown in the table.

added to the PEX reaction instead of dU^{Fc}TP, no peak around 0.4 V was detected (Figure 3). In the absence of DNA polymerase no primer extension took place. Accordingly, the resulting electrochemical response showed no Fc-specific peak regardless of the dNTP mix used. Heights of the peak G^{ox} were similar for pex^{AT}(U^{Fc}), the unlabeled pex^{AT}, as well as the unextended primer, in agreement with the fact that the total number of guanine residues was not changed in the PEX (Figure 3). This intrinsic DNA signal was further used as an internal control of the ON recovery from the magnetoseparation procedure and for normalization of the Fc tag signal.

Analogous results were obtained when dA^{Fc}TP was combined in the PEX reaction with unlabeled dTTP, but the A^{Fc} signal (peak A^{Fc}) appeared at a potential of 0.450 V, being positively shifted by ≈50 mV relative to the signal of dA^{Fc}TP (Figure 2B, Table 1). Hence, a considerable peak potential difference was retained between the A^{Fc} and U^{Fc} after their incorporation into DNA. Moreover, each of the modified nucleotides within the pex^{AT} product was electrochemically distinguishable from the respective dN^{Fc}TP.

Experiments with various ON templates revealed that all products involving the labeled nucleotides gave electrochemical signals due to the Fc tags. Potentials of the peaks yielded by ONs bearing U^{Fc} were always less positive than peak potentials yielded by ONs with A^{Fc} (see Table 1 for examples). Interestingly, peak potentials of both types of conjugates appeared to be influenced by nucleotide sequence, by number of the tags, and possibly by the type of neighboring base. The apparent redox potentials of different PEX products may be affected by the mode of adsorption and mutual interactions at the electrode surface (which in turn can be influenced by both nucleotide sequence,[15] as well as density of the Fc tags). Elucidation of these phenomena is a matter of ongoing research and will be published elsewhere.

Bioanalytical applications: The above results demonstrate that the dN^{Fc}TPs are applicable in PEX-based introduction of labeled nucleobase in DNA, and that incorporation of the A^{Fc} or U^{Fc} conjugates can easily and reliably be monitored by using a simple electrochemical technique and inexpensive carbon electrodes. These features make the presented approach promising for applications in bioanalysis and particularly in simple DNA "minisequencing"[16] and mapping techniques. For example, results shown in Figure 4A

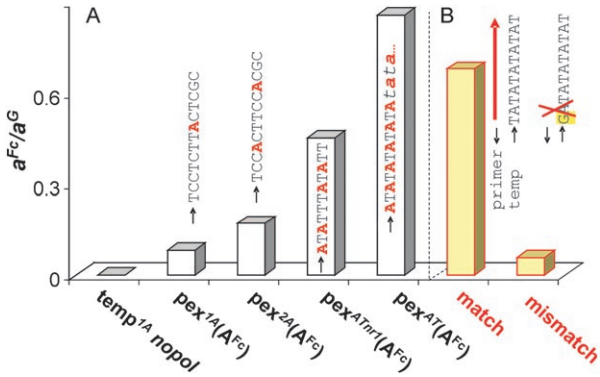


Figure 4. A) Effect of the number of A^{Fc} residues incorporated in PEX products on the intensity of the peak A^{Fc}. PEX: Klenow (exo-), dA^{Fc}TP+unlabeled dNTPs mix with respect to the given template. Synthesized stretches are inset; in pex^{AT}(A^{Fc}), the letters in lowercase indicate the expanded segment (see text and Supporting Information); "no pol", no DNA polymerase added. B) Detection of a single nucleotide mutation by using A^{Fc} as electroactive marker. PEX: DyNAzyme, dA^{Fc}TP+dTTP, templates temp^{AT} (match) or temp^{gAT} (mismatch: T at the first position replaced by G lacking its complementary counterpart in the dNTP mix). Ratios a^{Fc}/a^{G} (where a^{Fc} and a^{G} are areas of peak A^{Fc} due to the Fc label and of peak G^{ox} due to guanine, respectively; eight guanine residues are present in the primer stretch represented here by arrows) are plotted in the graph.

demonstrate that the intensity of the Fc tag specific signal depends on the number of A^{Fc} residues incorporated into the synthesized DNA stretch. In the order pex^{1A}(A^{Fc}), pex^{2A}-(A^{Fc}), and pex^{ATnrI}(A^{Fc}) (accommodating 1, 2, or 4 A^{Fc} residues, respectively) the intensity of the peak A^{Fc} was proportional to the number of Fc tags. Interestingly, for pex^{AT}(A^{Fc}) the peak A^{Fc} intensity was about tenfold higher than that for pex^{1A}(A^{Fc}) albeit the temp^{AT} sequence offered only six positions for A^{Fc} incorporation. PAGE revealed a remarkable expansion of the doublet $(AT)_n$ repeat sequence during the PEX reaction with dA^{Fc}TP and dTTP (see Figure 2 in the Supporting Information). Elongation of the synthesized stretch by up to ten nucleotides (when Klenow (exo-) was

used) was in agreement with the observed electrochemical signal increase. Thus, the proportion of a base (complementary to the $dN^{Fc}TP$ used) in a template DNA can be estimated from the electrochemical signal intensities. Such approach is potentially useful, for example, in mapping the abundance of particular nucleotides in specific DNA regions, as well as in studying repetitive sequences expansions.[17]

Searching for point mutations and sequence polymorphisms[16] is another area of prospective application of the modified nucleotides. Single-base substitutions can be detected via incorporation of a labeled nucleotide complementary at the mutated position. In addition, the possibility of signal amplification via incorporation of multiple electroactive tags suggests application of techniques based on termination of the PEX at the mutated site in the absence of a dNTP complementary to the expected mutation. As shown in Figure 4B, synthesis of the $A(TA)_n$ stretch in the presence of $dA^{Fc}TP + dTTP$ resulted in an intense Fc tag signal. When, however, the first T in the template stretch was replaced by G and the same dNTP mix was used, only a small signal (probably due to an accidental misincorporation of A^{Fc}) was obtained suggesting that the primer elongation could not proceed. An analogous principle can be used for detection of homonucleotide blocks (based on the low feasibility of incorporation of Fc-nucleobase conjugates at adjacent positions, see above).

Conclusion

Modified dNTPs (A or U) bearing Fc labels linked via a conjugate acetylene spacer were prepared by single-step aqueous-phase cross-coupling reactions of halogenated dNTPs with ethynylferrocene. The synthesis is very straightforward, efficient and avoids the use of protecting groups. The Fc-labeled dNTPs were good substrates for DNA polymerases and were efficiently incorporated into DNA by primer extension by using either Klenow (exo-) or DyNAzyme polymerases. Single-strand oligonucleotides bearing Fc labels were prepared by primer extension by using a biotinylated template followed by magnetoseparation on streptavidine-coated magnetic beads. Electrochemical analysis of the dNTPs and PEX products revealed significant differences in redox potentials of the Fc label bound either to U or to 7-deazaA and between isolated dNTPs and conjugates incorporated into DNA. Due to the fully conjugate acetylene linker, the ferrocene label responds well to electronic changes at the nucleobase and gives analytically useful changes in redox potentials.

This approach enables a facile construction of ferrocene-labeled DNA or single-strand oligonucleotides without unwanted side reactions (i.e., cyclization of Fc-ethynyl-U to furopyrimidines[5]). Incorporation of the labeled nucleobases can easily be monitored by using a simple electrochemical technique. These features make the new type of Fc labeling of nucleic acids suitable for applications in bioanalysis and particularly in simple DNA "minisequencing"[16] and mapping techniques. Incorporation of multiple tags in DNA results in a proportional signal amplification which can be used to estimate the abundance of a particular base in the template stretch, to monitor a repeat sequence expansion, as well as to detect a single nucleotide substitution. The bioanalytical applications outlined here will be subject of our further investigation.

Experimental Section

NMR spectra were measured on a Bruker DRX 500 (500, 125.8, and 202.3 MHz for ^1H, ^{13}C, and ^{31}P, respectively) in D_2O (referenced to dioxane as internal standard, $\delta_H = 3.75$ ppm, $\delta_C = 67.19$ ppm). Chemical shifts are given in ppm (δ scale), coupling constants (J) in Hz. Mass spectra were measured on an LCQ classic (Thermo–Finnigan) spectrometer by using ES. The H_2O/acetonitrile mixture was degassed in vacuo and stored under argon. Preparative HPLC separations were performed on a column packed with 10 μm of C18 reversed phase (Phenomenex, Luna C18(2)). The starting 7-iodo-7-deaza-dATP[11] and 5-iodo-dUTP[18] were prepared according to previously reported methods. All other chemicals were purchased from commercial suppliers.

7-Deaza-7-(ferrocene-1-yl-ethynyl)-2′-deoxyadenosine-5′-triphosphate ($dA^{Fc}TP$): A mixture of $H_2O/CH_3CN = 2{:}1$ (1 mL) followed by Et_3N (58 μL, 8 equiv) were added to an argon-purged flask containing 7-deaza-7-iodo-2′-deoxyadenosine-5′-triphosphate (50 mg, 0.053 mmol), ethynylferrocene (16 mg, 0.078 mmol, 1.5 equiv), and CuI (1 mg, 10 mol%). In a separate flask, $Pd(OAc)_2$ (0.6 mg, 5 mol%) and $P(Ph\text{-}SO_3Na)_3$ (7.5 mg, 5 equiv to Pd) were combined under argon and a mixture of $H_2O/CH_3CN = 2{:}1$ (0.5 mL) was added. After dissolution of the solids, the catalyst solution was added to the reaction mixture through a septum and the mixture was stirred at 70 °C for 1 h. The solvent was evaporated under vacuum and the residue was dissolved in water. The insoluble material was filtered off and the product was purified by using RP HPLC. The final product was freeze-dried from aqueous solution to obtain the desired triphosphate as dihydrate and tris-triethylammonium salt (27 mg, 48%). ^1H NMR (500 MHz, D_2O, $ref_{dioxane} = 3.75$ ppm): $\delta = 2.42$–2.60 (m, 2H; H-2′), 4.23–4.37 (m, 8H; H-4′,5′ and cp), 4.12 (m br, 2H; H-3″,4″), 4.58 and 4.64 (2 × m br, 2H; H-2″,5″), 4.70 (m br, 1H; H-3′), 6.48 (t br, $J_{1',2'} = 5.9$ Hz, 1H; H-1′), 7.72 (s, 1H; H-8), 8.23 ppm (s br, 1H; H-2); ^{13}C NMR (125.7 MHz, D_2O, $ref_{dioxane} = 69.3$ ppm): $\delta = 42.77$ (CH_2–2′), 65.6 (C-1″), 68.4 (d, $J(C,P) = 4$ Hz, CH_2–5′), 72.48 and 72.51 (CH-3″,4″), 72.8 (CH-cp), 73.7 (CH-3′), 74.2 and 74.3 (CH-2″,5″), 78.4 (*C*≡C-Fc), 86.6 (CH-1′), 88.3 (d, $J(C,P) = 9$ Hz, CH-4′), 96.3 (C≡*C*-Fc), 102.0 (C-7), 104.3 (C-5), 129.8 (CH-8), 146.3 (CH-2), 148.5 (C-4), 153.6 ppm (C-6); ^{31}P NMR (202.3 MHz, D_2O): −22.33 (t, $J = 23$, 20 Hz, P_β), −10.46 (d, $J = 20$ Hz, P_α), −10.10 ppm (d, $J = 23$ Hz, P_γ); UV/Vis (H_2O): λ_{max} (ε) = 232 (109 849), 281 (89 393), 440 nm (3788); MS TOF ES (positive-ion mode): m/z (%): 721.53 (30) [M^++Na], 801.46 (70) [M^++Et_3N], 902.64 (100) [M^++2 × Et_3N].

5-(Ferrocene-1-yl-ethynyl)-2′-deoxyuridine-5′-triphosphate ($dU^{Fc}TP$): A mixture $H_2O/CH_3CN = 2{:}1$ (1 mL) followed by Et_3N (58 μL, 8 equiv) were added to an argon-purged flask containing 5-iodo-2′-deoxyuridine-5′-triphosphate (47 mg, 0.053 mmol), ethynylferrocene (22 mg, 0.106 mmol, 2 equiv), and CuI (3 mg, 30 mol%). In a separate flask $Pd(OAc)_2$ (1.2 mg, 10 mol%) and $P(Ph\text{-}SO_3Na)_3$ (15 mg, 5 equiv to Pd) were combined under argon and a mixture of $H_2O/CH_3CN = 2{:}1$ (0.5 mL) was added. After dissolution of the solids, the catalyst solution was added to the reaction mixture through a septum and the mixture was stirred at 65 °C for 1 h. The solvent was evaporated under vacuum and the residue was dissolved in water. The insoluble material was filtered off and the product was purified by using RP HPLC. The final product was freeze-dried from aqueous solution to obtain the desired triphosphate as dihydrate and tris-triethylammonium salt (22 mg, 42%). ^1H NMR (500 MHz, D_2O, $ref_{dioxane} = 3.75$ ppm): $\delta = 2.36$ (bdt, $J_{gem} = 13.8$ Hz, *J*-

(2′b,1′)=*J*(2′b,3′)=6.9 Hz, 1H; H-2′b), 2.65 (bdt, J_{gem}=13.8 Hz, *J*-(2′a,1′)=*J*(2′a,3′)=5.3 Hz, 1H; H-2′a), 4.23–4.44 (m, 8H; H-4′,5′ and cp), 4.62 (m br, 1H; H-3′), 4.67 (m br, 2H; H-2″,5″), 5.04 (m br, 2H; H-3″,4″), 6.38 (t br, *J*(1′,2′)=6.9, 5.3 Hz, 1H; H-1′), 8.69 ppm (s, 1H; H-6); ^{13}C NMR (125.7 MHz, D_2O, $ref_{dioxane}$=69.3 ppm): δ=43.0 (CH_2–2′), 67.8 (CH_2–5′), 69.4 (CH-3″,4″), 72.7 (CH-3′), 72.9 (CH-cp), 73.2 (CH-2″,5″), 75.1 (*C*≡C-Fc), 88.8 (d, *J*(C,P)=9, CH-4′), 91.0 (CH-1′), 94.3 (C≡**C**-Fc), 113.5 (C-5), 139.0 (CH-6), 158.7 (C-2), 174.1 ppm (C-4), C-1″ not observed; ^{31}P NMR (202.3 MHz, D_2O): −22.61 (t, *J*=19, 18 Hz, P_β), −10.85 (d, *J*=19 Hz, P_α), −10.32 ppm (d, *J*=18 Hz, P_γ); UV/Vis (H_2O): λ_{max} (ε)=253 (86885), 282 (83607), 340 (58197), 468 nm (7377) (sh); MS ES (negative-ion mode): *m*/*z* (%): 675.0 (100) [*M*].

Primer extension, purification, and analysis of the PEX products

Materials: Synthetic ONs were purchased from VBC genomics (Austria): Primer: 5′-CAT GGG CGG CAT GGG-3′; templates: 5′-**TAT ATA TAT AT***C CCA TGC CGC CCA TG*-3′ (temp^{AT}); 5′-**TAT ATA TAT AG**C CCA TGC CGC CCA TG-3′(temp^{gAT}); 5′-**AAT ATA AAT AT**C CCA TGC CGC CCA TG-3′ (temp^{ATnr1}); 5′-**TTA TAT TTA AT**C CCA TGC CGC CCA TG-3′ (temp^{ATnr2}); 5′-**GCG AGG AAG TGG AG**C CCA TGC CGC CCA TG-3′ (temp^{1A}); 5′-**GCG TGG AAG TGG AG**C CCA TGC CGC CCA TG-3′ (temp^{2A}); 5′-**TCT CTC TCT CT**C CCA TGC CGC CCA TG-3′ (temp^{CT}); 5′-**TGT GTG TGT GT**C CCA TGC CGC CCA TG-3′ (temp^{GT}); 5′-**AGA GAG AGA GA**C CCA TGC CGC CCA TG-3′ (temp^{AG}); 5′-**ACA CAC ACA CA**C CCA TGC CGC CCA TG-3′ (temp^{AC}) (segments forming a duplex with the primer are underlined, the replicated segments are in bold). Templates used in experiments involving the DB_{stv} magnetoseparation procedure were biotinylated at their 5′ ends. Acronyms used in this work for the PEX products are analogous to those introduced for the templates (e.g., a PEX product pex^{AT} was synthesized on the temp^{AT} template, etc.) Dynabeads M-270 streptavidin (DB_{stv}) were obtained from Dynal A.S. (Norway), Klenow Fragment (3′→5′ exo-) and T4 polynucleotide kinase from New England Biolabs (Great Britain), DyNAzyme II DNA Polymerase from Finnzymes (Finland), unmodified nucleoside triphosphates (dATP, dTTP, dCTP, and dGTP) from Sigma and α-^{32}P-ATP from MP Empowered Discovery (USA). Other chemicals were of analytical grade.

Primer extension: The primer (0.7 μM) was mixed with a template ON (0.7 μM), dNTPs (125 μM each; composition of the dNTP is specified in the text and Figure legends for individual experiments) and a DNA polymerase (1–2.5 U per sample). For polyacrylamide gel electrophoresis (PAGE) experiments, the primer was ^{32}P-prelabeled at its 5′ end and unmodified templates were used. For electrochemical analysis involving the magnetoseparation procedure, unlabeled primer and biotinylated templates were used. Reactions with Klenow (exo-) polymerase were conducted at 37 °C for 45 min, those with DyNAzyme II at 60 °C for 30 min.

DB_{stv} magnetoseparation procedure: The PEX products were captured at DB_{stv} via biotin tags tethered to the 5′ ends of the template strands. Then, 50-μL aliquots of the PEX reaction mixtures were added to the DB_{stv} (25 μL of the stock suspension washed twice by 100 μL of 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4 (buffer H)). The mixtures were incubated on a shaker for 30 min at 20 °C. Then the beads were subsequently washed three times by 100 μL of PBS (0.14 M NaCl, 3 mM KCl, 4 mM sodium phosphate pH 7.4) with 0.01 % Tween 20, three times by 100 μL of the buffer H and resuspended in deionized water (50 μL). The extended primer strands were released by heating at 75 °C for 2 min. Each medium exchange was performed by using a magnetoseparator (Dynal, Norway). Prior to the ex situ electrochemical measurements, NaCl was added to the samples (final concentration 0.2 M).

Polyacrylamide gel electrophoresis: The PEX products were mixed with loading buffer (80 % formamide, 10 mM EDTA, 1 mg mL^{-1} xylene cyanol, 1 mg mL^{-1} bromphenol blue) and subjected to electrophoresis in 15 % denaturing polyacrylamide gel containing 1xTBE buffer (pH 8) and 7 M urea at 25 W for 50 min. Gels were dried, autoradiographed, and visualized by using Phosphorimager Storm.

Electrochemical analysis: The PEX oligonucleotide products were analyzed by using ex situ (adsorptive transfer stripping) square-wave voltammetry (SWV). The PEX products were accumulated at the basal-plane pyrolytic graphite electrode (PGE; prepared and pretreated as described[19]) surface from 5 μL aliquots containing 0.2 M NaCl for 60 s. Then the electrode was rinsed by deionized water and was placed into the electrochemical cell. Electrochemical responses of free Fc, dA^{Fc}TP, and dU^{Fc}TP (Figure 2) were measured in a conventional in situ mode (with the analyte dissolved in background electrolyte). SWV settings: initial potential −1.0 V, final potential +1.5 V, pulse amplitude 25 mV, frequency 200 Hz, potential step 5 mV. The measurements were performed at ambient temperature in 0.1 M Tris, 0.2 M NaCl, pH 7.3 by using an Autolab analyzer (EcoChemie, The Netherlands) in a three-electrode setup (with the PGE as working electrode, Ag/AgCl/3 M KCl as reference, and platinum wire as counter-electrode). Except for the curves shown in Figure 2A and in Figure 4 in the Supporting Information, the voltammograms were baseline corrected by means of a moving average algorithm (GPES 4 software, EcoChemie) (for further comments see the Supporting Information).

Acknowledgements

This work is a part of the research projects Z4 055 0506 and Z5 004 0507. It was supported by EC STREP Project CIDNA (to M.H.), by the Grant Agency of the Czech Republic (203/05/0043 to M.H., L.H., and M.F.), by the Ministry of Education (LC512 to M.H., LC06035 to M.F.) and by Gilead Sciences, Inc. (Foster City, CA, USA).

[1] M. J. Heller, *Annu. Rev. Biomed. Eng.* **2002**, *4*, 129–153.
[2] a) E. Palecek, F. Jelen in *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors For Genomics and Proteomics* (Eds.: E. Palecek, F. Scheller, J. Wang), Elsevier, Amsterdam, **2005**, pp. 74–174; b) J. Wang, in *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors For Genomics and Proteomics* (Eds.: E. Palecek, F. Scheller, J. Wang), Elsevier, Amsterdam, **2005**, 175–194; c) J. Labuda, M. Fojta, F. Jelen, E. Palecek in *Encyclopedia of Sensors, Vol. 3, E–F* (Eds.: C. A. Grimes, E. C. Dickey, M. V. Pishko), American Scientific Publishers, Stevenson Ranch, CA, USA, **2006**, pp. 201–228; d) M. Fojta, *Collect. Czech. Chem. Commun.* **2004**, *69*, 715–747.
[3] D. R. Van Staveren, N. Metzler-Nolte, *Chem. Rev.* **2004**, *104*, 5931–5985.
[4] a) C. J. Yu, H. Wang, Y. J. Wan, H. Yowanto, J. C. Kim, L. H. Donilon, C. L. Tao, M. Strong, Y. C. Chong, *J. Org. Chem.* **2001**, *66*, 2937–2942; b) C. J. Yu, Y. J. Wan, H. Yowanto, J. Li, C. L. Tao, M. D. James, C. L. Tan, G. F. Blackburn, T. J. Meade, *J. Am. Chem. Soc.* **2001**, *123*, 11155–11161.
[5] a) C. J. Yu, H. Yowanto, Y. J. Wan, T. J. Meade, Y. Chong, M. Strong, L. H. Donilon, J. F. Kayyem, M. Gozin, G. F. Blackburn, *J. Am. Chem. Soc.* **2000**, *122*, 6767–6768; b) A. R. Pike, L. C. Ryder, B. R. Horrocks, W. Clegg, M. R. J. Elsegood, B. A. Connolly, A. Houlton, *Chem. Eur. J.* **2002**, *8*, 2891–2899.
[6] a) D. A. Di Giusto, W. A. Wlassoff, S. Geisebrecht, J. J. Gooding, G. C. King, *J. Am. Chem. Soc.* **2004**, *126*, 4120–4121; b) D. A. Di Giusto, W. A. Wlassoff, S. Geisebrecht, J. J. Gooding, G. C. King, *Angew. Chem.* **2004**, *116, 2869–2872; Angew. Chem. Int. Ed.* **2004**, 43, 2809–2812; *Angew. Chem. Int. Ed.* **2004**, *43*, 2809–2812; c) W. A. Wlassoff, G. C. King, *Nucleic Acids Res.* **2002**, *30*, e58; d) S. S. W. Yeung, T. M. H. Lee, I.-M. Hsing, *J. Am. Chem. Soc.* **2006**, *128*, 13374–13375; e) F. Patolsky, Y. Weizmann, I. Willner, *J. Am. Chem. Soc.* **2002**, *124*, 770–772.
[7] M. Hocek, P. Štěpnička, J. Ludvík, I. Císařová, I. Votruba, D. Řeha, P. Hobza, *Chem. Eur. J.* **2004**, *10*, 2058–2066.
[8] A. R. Pike, L. C. Ryder, B. R. Horrocks, W. Clegg, B. A. Connoly, A. Houlton, *Chem. Eur. J.* **2005**, *11*, 344–353.
[9] a) K. Sakthivel, C. F. Barbas III, *Angew. Chem.* **1998**, *110*, 2998–3002; *Angew. Chem. Int. Ed.* **1998**, *37*, 2872–2875; b) H. Sawai, A. N. Ozaki, F. Satoh, T. Ohbayashi, M. M. Masud, H. Ozaki, *Chem. Commun.* **2001**, 2601–2605; c) T. Gourlain, A. Sidorov, N. Mignet,

S. J. Thorpe, S. E. Lee, J. A. Grasby, D. M. Williams, *Nucleic Acids Res.* **2001**, *29*, 1898–1905; d) H. Sawai, A. Ozaki-Nakamura, M. Mine, H. Ozaki, *Bioconjugate Chem.* **2002**, *13*, 309–316; e) M. M. Masud, A. Ozaki-Nakamura, M. Kuwahara, H. Ozaki, H. Sawai, *ChemBioChem* **2003**, *4*, 584–588; f) M. Kuwahara, J. Nagashima, M. Hasegawa, T. Tamura, R. Kitagata, K. Hanawa, S. Hososhima, T. Kasamatsu, H. Ozaki, H. Sawai, *Nucleic Acids Res.* **2006**, *34*, 5383–5394; g) S. Jäger, M. Famulok, *Angew. Chem.* **2004**, *116*, 3399–3403; *Angew. Chem. Int. Ed.* **2004**, *43*, 3337–3340; h) S. Jäger, G. Rasched, H. Kornreich-Leshem, M. Engeser, O. Thum, M. Famulok, *J. Am. Chem. Soc.* **2005**, *127*, 15071–15082; i) M. Kuwahara, K. Hanawa, K. Ohsawa, R. Kitagata, H. Ozaki, H. Sawai, *Bioorg. Med. Chem.* **2006**, *14*, 2518–2526; j) A. Shoji, T. Hasegawa, M. Kuwahara, H. Ozaki, H. Sawai, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 776–779; k) M. Matsui, Y. Nishiyama, S. Ueji, Y. Ebara, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 456–460.

[10] P. Čapek, R. Pohl, M. Hocek, *Org. Biomol. Chem.* **2006**, *4*, 2278–2284.

[11] P. Čapek, H. Cahová, R. Pohl, M. Hocek, C. Gloeckner, A Marx, *Chem. Eur. J.* **2007**, *13*, 6196–6203.

[12] L. H. Thoresen, G.-S. Jiao, W. C. Haaland, M. L. Metzker, K. Burgess, *Chem. Eur. J.* **2003**, *9*, 4603–4610.

[13] a) J. Wang, G. Liu, A. Merkoci, *J. Am. Chem. Soc.* **2003**, *125*, 3214–3215; b) M. Fojta, L. Havran, R. Kizek, S. Billova, E. Paleček, *Biosens. Bioelectron.* **2004**, *20*, 985–994; c) M. Fojta, P. Kostecka, M. Trefulka, L. Havran, E. Paleček, *Anal. Chem.* **2007**, *79*, 1022–1029; d) M. Masarik, K. Cahova, R. Kizek, E. Paleček, M. Fojta, *Anal. Bioanal. Chem.* **2007**, *388*, 259–270.

[14] M. Vrábel, M. Hocek, L. Havran, M. Fojta, I. Votruba, B. Klepetářová, R. Pohl, L. Rulíšek, L. Zendlová, P. Hobza, I. Shih, E. Mabery, R. Mackman, *Eur. J. Inorg. Chem.* **2007**, 1752–1769.

[15] L. Trnkova, F. Jelen, I. Postbieglova, *Electroanalysis* **2006**, *18*, 662–669.

[16] a) S. Brazill, N. E. Hebert, W. G. Kuhr, *Electrophoresis* **2003**, *24*, 2749–2757; b) A. C. Syvanen, *Hum. Mutat.* **1999**, *13*, 1–10.

[17] M. Fojta, L. Havran, M. Vojtíšková, E. Paleček, *J. Am. Chem. Soc.* **2004**, *126*, 6532–6533.

[18] T. Kovacs, L. Ötvös, *Tetrahedron Lett.* **1988**, *29*, 4525–4528.

[19] a) M. Fojta, L. Havran, R. Kizek, S. Billova, *Talanta* **2002**, *56*, 867–874; b) M. Fojta, L. Havran, S. Billova, P. Kostecka, M. Masarik, R. Kizek, *Electroanalysis* **2003**, *15*, 431–440.

Received: August 10, 2007
Published online: September 25, 2007

