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Base-Modified DNA Labeled by $[Ru(bpy)_3]^{2+}$ and $[Os(bpy)_3]^{2+}$ Complexes: Construction by Polymerase Incorporation of Modified Nucleoside Triphosphates, Electrochemical and Luminescent Properties, and Applications

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Abstract: Modified 2′-deoxynucleoside triphosphates (dNTPs) bearing $[Ru(bpy)_3]^{2+}$ and $[Os(bpy)_3]^{2+}$ complexes attached via an acetylene linker to the 5-position of pyrimidines (C and U) or to the 7-position of 7-deazapurines (7-deaza-A and 7-deaza-G) have been prepared in one step by aqueous cross-couplings of halogenated dNTPs with the corresponding terminal acetylenes. Polymerase incorporation by primer extension using Vent (exo-) or Pwo polymerases gave DNA labeled in specific positions with Ru^{2+} or Os^{2+} complexes. Square-wave voltammetry could be efficiently used to detect these labeled nucleic acids by reversible oxidations of $Ru^{2+/3+}$ or $Os^{2+/3+}$. The redox potentials of the Ru^{2+} complexes (1.1–1.25 V) are very close to that of G oxidation (1.1 V), while the potentials of Os^{2+} complexes (0.75 V) are sufficiently different to enable their independent detection. On the other hand, Ru^{2+}-labeled DNA can be independently analyzed by luminescence. In combination with previously reported dNTPs bearing ferrocene, aminophenyl, and nitrophenyl tags, the Os-labeled dATP has been successfully used for "multicolor" redox labeling of DNA and for DNA minisequencing.

Keywords: DNA polymerase · electrochemistry · oligonucleotides · osmium · ruthenium

Introduction

DNA biosensors and chips are broadly utilized in the life sciences.[1] 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 the sensitivity and specificity of sequence-specific electrochemical DNA sensing.[3] Complexes of bipyridine (bpy) ligands with transition metals possess unique electrochemical and photophysical properties[4] and may thus be used for electrochemical or luminescent labeling of biomolecules. Some transition metal complexes (as DNA intercalators/insertors[5] or covalent end-labels[6]) have been extensively used as luminescent and electroactive DNA labels for bioanalytical applications and charge-transfer studies. The attachment of probes based on metal complexes to nucleobases via conjugated linkers[7] should increase the efficiency of charge transfer and thus enhance sensitivity. Covalently bound conjugates of pyrimidine nucleotides and phenanthroline complexes of Ru and Os have been studied by Tor et al.[8,9] as lu-

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minescent probes for DNA hybridization and charge transfer through DNA. 2′-Deoxyuridine phosphoramidites bearing Ru^{2+} and Os^{2+} complexes attached via acetylene units at the 5-position have been prepared and chemically incorporated into oligonucleotides by solid-phase synthesis.[8] In addition, an Ru^{2+}-containing complex of dUTP with *N*-substituted hydroxamate as a non-conjugated linker has been prepared by a multistep procedure and enzymatically incorporated into 22- and 27-mer oligonucleotides by Klenow fragment polymerase.[9] The corresponding labeling of purines was not studied until recently, when we reported on the synthesis and electrochemistry of model 9-benzyladenine derivatives bearing oligopyridine ligands, or the Ru or Os complexes thereof, attached at the 8-position through conjugated phenylene or acetylene tethers.[10] These tethers were designed to transmit electronic changes from the nucleobase to the electroactive label. Subsequently, we reported the synthesis of the 2′-deoxyadenosine nucleoside bearing bpy ligands and the corresponding Ru complexes at the 8-position.[11] However, our follow-up study on the chemical incorporation of the corresponding protected 8-substituted 2′-deoxyadenosine phosphoramidites on a solid support was unsuccessful due to very low coupling yields.

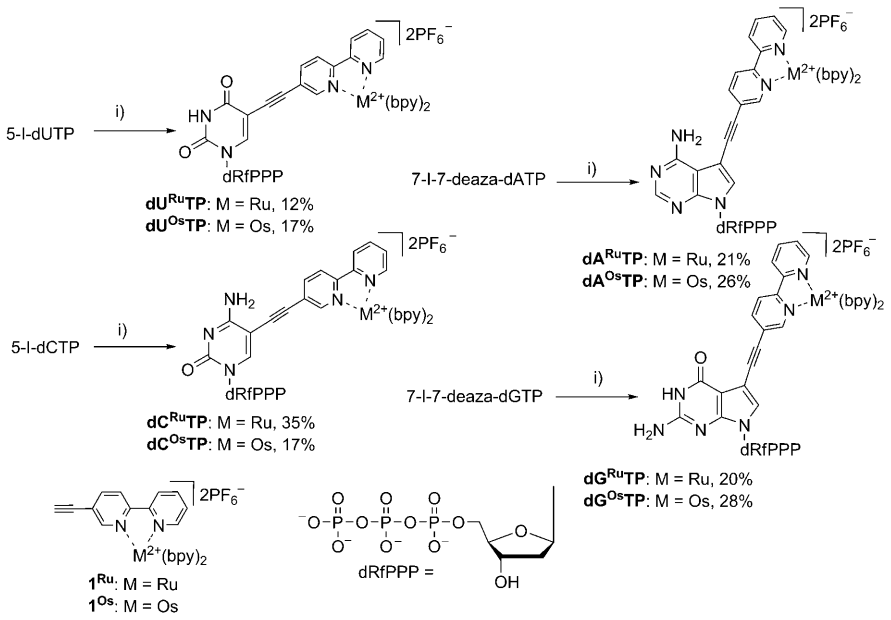
An alternative strategy for the construction of functionalized nucleic acids is based on polymerase incorporations of base-modified nucleoside triphosphates (dNTPs).[12] It can even be combined with cross-coupling reactions of dNTPs,[13] and we have recently used this novel approach[14] for the synthesis of DNA bearing amino acids,[15] ferrocenes,[16] and aminophenyl and nitrophenyl groups.[17] Our studies[16,17] also demonstrated that the attachment of the redox label (ferrocene or amino/nitro groups) via a conjugated acetylene or phenylene linker enables electronic communication between the nucleobase and label, and that the redox potential responds to the incorporation to DNA. On the other hand, we[14,18] and others[12c] have shown that 8-substituted purine dNTPs are poor substrates for DNA polymerases (presumably due to their preference for the *syn*-conformation and steric hindrance between the substituent and DNA backbone) and should be replaced by 7-substituted 7-deazapurine dNTPs. Therefore, our further efforts have been focused on the attachment of $Ru(bpy)_3$ complexes to the 7-position of 7-deaza-2′-deoxyadenosine and we have recently reported the synthesis and electrochemical and photophysical properties of these nucleosides.[19] That study also showed that the redox potentials of the Ru complexes are largely independent of the type of bpy ligand and the position of the conjugated linkage, but that, on the other hand, only one type of Ru complex (ethynyl linker attached to the 3-position of the bpy ligand) offers useful levels of luminescence.[19] Therefore, we have now focused exclusively on complexes of this type for DNA labeling, and we report herein on the synthesis of all four modified dNTPs bearing either Ru^{2+} or Os^{2+} complexes, their polymerase incorporations, electrochemical properties, and bioanalytical applications.

Results and Discussion

Synthesis of modified dNTPs: In our initial studies,[10,11] we found that the best strategy for the attachment of $M(bpy)_3$-type complexes to nucleobases is to perform the cross-coupling reactions directly with the whole metal-containing building block (rather than to attach the ligand first and then perform the complexation, which requires harsh conditions, as a subsequent step). Therefore, our building blocks of choice were the terminal acetylene derivatives of the complexes $[M(bpy)_2(5\text{-ethynyl-}2,2'\text{-bpy})]^{2+}(PF_6^-)_2$ $\mathbf{1^{Ru}}$ (M = Ru)[10] or $\mathbf{1^{Os}}$ (M = Os). Sonogashira cross-couplings of such acetylenes with halogenated dNTPs could then be performed in aqueous media in analogy to our previous studies on nucleosides,[11,19] provided that due attention was paid to the very low stability of the dNTPs.

Thus, the cross-coupling reactions of $\mathbf{1^{Ru}}$ or $\mathbf{1^{Os}}$ with 5-iodo-2′-deoxyuridine 5′-triphosphate (5-I-dUTP),[20] 5-iodo-2′-deoxycytidine 5′-triphosphate (5-I-dCTP),[17] 7-iodo-7-deaza-2′-deoxyadenosine 5′-triphosphate (7-I-7-deaza-dATP),[15] and 7-iodo-7-deaza-2′-deoxyguanosine 5′-triphosphate (7-I-7-deaza-dGTP)[21] were performed in the presence of $Pd(OAc)_2$, TPPTS, CuI, and Et_3N in H_2O/CH_3CN (2:1) at 65 °C within 1 h to afford the desired Ru^{2+}- and Os^{2+}-containing nucleoside triphosphates (**dN^{M}TP**s) in moderate yields of 12–35 % (Scheme 1). The relatively low isolated yields of the modified dNTPs may be attributed to partial hydrolysis of the starting and final dNTPs during the reaction and further decomposition of the latter during isolation by RP-HPLC. Therefore, the reactions always had to be terminated within 1 h at most (even if the conversion was not yet complete) and then the whole reaction mixture had to be immediately concentrated, applied to an HPLC column, and eluted, and the products had to be promptly lyophilized to obtain relatively stable solid material. When dissolved in water, the products were very quickly hydrolyzed, and even frozen aqueous solutions stored at −20 °C decomposed within a few weeks. However, despite the rather moderate yields, this approach was very efficient and straightforward since it consisted of just one single step performed on unprotected substrates and the isolated yields (and amounts) of the modified dNTPs were sufficient for characterization and incorporation experiments. Moreover, a complete set of dNTPs from all four nucleobases (U, C, A, and G), in each case bearing both Ru and Os complexes, was prepared in order to study the influence of the nucleobase on the incorporation and redox potentials. It should be mentioned here that the $Ru(bpy)_3/Os(bpy)_3$ complexes are chiral and their attachment to homochiral D-nucleoside triphosphates results in the formation of mixtures of non-separable (chromatographically homogeneous) diastereoisomers. These diastereomeric mixtures were used in all follow-up biochemical and electrochemical experiments.

Polymerase incorporation of modified dNTPs: Weizman and Tor previously reported[9] the incorporation of dUTP bearing an $[Ru(acac)(bpy)_2]$ complex attached via a long



Scheme 1. i) $\mathbf{1}^{Ru}$ or $\mathbf{1}^{Os}$, $Pd(OAc)_2$, TPPTS, CuI, Et_3N, H_2O/CH_3CN 2:1, 1 h, 70 °C.

flexible linker to the 5-position into 22- and 27-mers (1 or 4 modifications) by Klenow fragment (exo-) polymerase. Our previous studies on other 5-modified dUTPs and dCTPs and on 7-modified 7-deaza-dATPs showed that Klenow polymerase could indeed be successfully used for most incorporations of such modifications, but that they are often accompanied by some misincorporations.[16,17] On the other hand, thermostable polymerases (DyNAzyme, Vent (exo-), or Pwo) typically gave more reliable incorporation with higher fidelity, but, in some cases, problems were observed when trying to incorporate two modified dNTPs in adjacent positions.[16,17] Therefore, we have tested all four enzymes for their efficacy in the incorporation of the title **dN^{M}TP**s. In view of the presence of the very bulky and charged metal complexes attached via a rigid acetylene linker, it was expected to be quite demanding for the polymerases to recognize these dNTPs as substrates, to incorporate them, and also to further extend the strand with another natural or modified dNTP.

The incorporation was tested on a 31-mer template temp^{rnd16} (Table 1) requiring four incorporations of modified **dN^{M}TP**s for each type of nucleobase using all four of the abovementioned polymerases (Klenow, DyNAzyme, Vent (exo-), and Pwo). While the use of Klenow fragment at 37 °C or DyNAzyme at 60 °C gave incomplete incorporations (similar to negative control experiments), Vent (exo-) (Figure 1) and Pwo (see Supporting Information) efficiently gave full-length oligonucleotides (ONs) for each of the modified **dN^{M}TP**s. The mobility of these functionalized ONs on PAGE was lower due to the additional mass and positive charge of the metal-containing labels. Incorporations of **dN^{M}TP**s using other templates (Table 1) were also successfully performed. Preliminary kinetic studies (see Supporting Information) with two examples of modified **dN^{M}TP**s showed that they were incorporated almost as quickly as natural dNTPs (the extension was virtually complete within 2–5 min). Nevertheless, the primer extension (PEX) reaction mixtures were typically incubated for 30 min to ensure full extension. The presence of the metal complexes in the PEX products was confirmed by electrochemical and/or luminescence measurements (see below). Similar to previous observations for other labeled dNTPs,[16,17] PEX reactions leading to the incorporation of the conjugate bases at adjacent positions typically showed early termination. Rather difficult addition of another modified nucleotide to PEX products already bearing a 3′-terminal modification has been observed

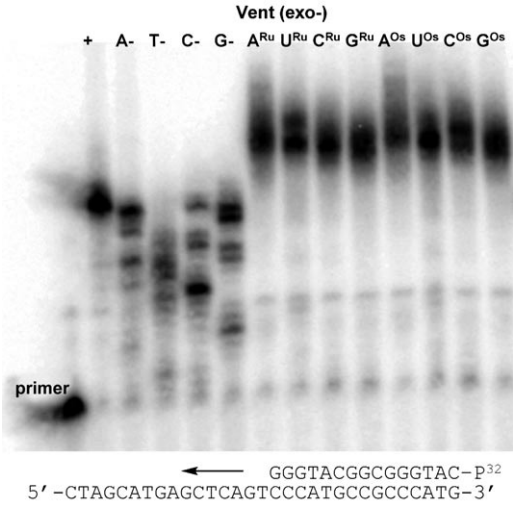


Figure 1. Denaturing PAGE analysis of incorporations of **dN^{M}TP**s using Vent (exo-) polymerase and temp^{rnd16}. Composition of the dNTP mixes and nucleotide labeling are as follows: "+" positive control (dATP, dTTP, dCTP, and dGTP), negative control experiments N− (absence of each one natural dNTP), **N^{M}** (**dN^{M}TP** + 3 other natural dNTPs).

Table 1. Primers and templates used for primer extension.

prim^{rnd}	5′-CATGGGCGGCATGGG-3′
prim^{noG}	5′-TACTCATCATATCAA-3′
temp^{rnd16}	5′-**CTAGCATGAGCTCAGT***CCCATGCCGCCCATG*-3′
temp^{C}	5′-**CCCG***CCCATGCCGCCCATG*-3′
temp^{U}	5′-**CCCA***CCCATGCCGCCCATG*-3′
temp^{A}	5′-**CCCT***CCCATGCCGCCCATG*-3′
temp^{noG}	5′-**AATATAAATATA***TTGATATGATGAGTA*-3′

for all combinations of two different **dN^{M}TP**s (see Supporting Information for examples), as well as for blocks of labeled nucleotides of the same type (see below).

As mentioned previously, the **dN^{M}TP**s were used as mixtures of diastereoisomers (due to the chiral $M(bpy)_3$ complexes) and it is possible that the polymerase may partly discriminate between them and incorporate one of them more frequently than the other. However, the CD spectra of the PEX products (see Supporting Information) did not give sufficient information to discern this and the limited amount of material produced by PEX does not allow for any other relevant analysis. The modified **dN^{M}TP**s were used in large excess so as to ensure that, even if only one diastereoisomer were incorporated, fully extended PEX products would still be obtained. Moreover, the chirality may have hardly any effect on the redox potential of the complex (as a DNA redox label). Therefore, we have not further studied the diastereoselectivity of the polymerase incorporations.

In the template (temp) ONs, the segments forming the duplex with the primer are italic in Table 1, and the replicated segments are in bold. For magnetic separation of the extended primer strands, the templates were 5′-end biotinylated. Acronyms used in the text for primer extension products are analogous to those introduced for the templates (e.g., a PEX product pex^{rnd16} was synthesized on temp^{rnd16} template).

Electrochemistry of the dNTP conjugates: Using square-wave voltammetry (SWV) with a pyrolytic graphite electrode (PGE),[16,17,22] the electrochemical responses of the modified dNTPs were studied (Figure 2). In 0.2 M NaCl/0.1 M Tris at pH 7.4, all of the Os complexes produced a well-developed reversible peak due to the $Os^{2+/3+}$ redox process at potentials between 0.73 and 0.75 V (Figure 2a, Table 2; the reversibility of the Os redox process is demonstrated in the Supporting Information, Figure S4). Analogous signals corresponding to the $Ru^{2+/3+}$ redox couple were observed with the **dN^{Ru}TP**s, close to 1.16 V (for pyrimidine conjugates) or 1.21 V (for 7-deazapurine conjugates; Figure 2c, Table 2). In addition to peaks corresponding to the reversible redox processes of the metals, the 7-deazapurine conjugates yielded irreversible anodic signals at potentials of around 0.75 V (G conjugates) or 1.0 V (A conjugates), which may be attributed to electrochemical oxidation of the 7-deazapurine moieties. For **dG^{Os}TP**, the latter signal was overlapping with the osmium peak (Figure 2a). No such peaks were observed with the labeled pyrimidines. Unlabeled 7-deazapurine dNTP derivatives produced irreversible anodic peaks at 0.60 V (G) or 0.93 V (A), that is, about 100 mV less positive than those of the corresponding Os- or Ru-labeled dNTPs (Figure 2 and Table 3). Thus, conjugation of the 7-deazapurines with the metal complexes made their oxidation more difficult. Compared to natural dGTP and dATP, the respective (unsubstituted) 7-deazapurine dNTPs were oxidized at potentials about 330 mV less positive (Table 3), making the oxidation signals useful for distinguishing between the natural and 7-deazapurines[23] (albeit the oxidation potential of 7-deaza-dATP was found to be coincident with that of dGTP, which may complicate analysis of DNAs containing both types of bases).

Analogous SWV measurements were also performed in 0.2 M sodium acetate at pH 5.0 (used as an optimum background electrolyte for measurements of labeled DNA). The $M^{2+/3+}$ redox potentials were found to be little influenced by the medium (Table 2). On the other hand, the oxidation peaks of the dNTP 7-deazapurines in the acetate buffer were shifted by about 80–120 mV to more positive potentials (compared with the same peaks observed in NaCl/Tris at pH 7.4; see Table 3 and Figure S6). Using the $Os^{2+/3+}$ SWV signal, the **dN^{Os}TP**s were easily detectable at 10^{-8} M concentrations after relatively short accumulation times (2–3 min, see Supporting Information).

Electrochemical analysis of Os- and Ru-labeled PEX products: PEX products pex^{rnd16} bearing the Os or Ru labels were synthesized using the temp^{rnd16} template, Vent (exo-) polymerase, and dNTP mixes containing one of the **dN^{M}TP** conjugates complemented with the remaining three natural **dNTP**s. The resulting products were purified using Qiagen Nucleotide Removal kit and analyzed by means of ex situ SWV. Typical SWV responses (measured in 0.2 M acetate at pH 5.0) resulting from the PEX experiments are shown in Figure 3a). The pex^{rnd16}(**A^{Os}**) synthesized in the presence of **dA^{Os}TP** yielded, in addition to signals due to the oxidation of guanine and adenine residues (peaks G^{ox} and A^{ox} at 1.09 and 1.37 V, respectively), a well-developed peak at 0.70 V, which could be attributed to the incorporated **A^{Os}**. The latter signal was not observed for unlabeled pex^{rnd16} (resulting from PEX with natural dNTP mix) or for the product of the negative PEX reaction (the dNTP mix contained **dA^{Os}TP** but no DNA polymerase was added). The analogous PEX products pex^{rnd16}(**G^{Os}**), pex^{rnd16}(**C^{Os}**), and pex^{rnd16}(**U^{Os}**), synthesized with mixes containing **dG^{Os}TP**, **dC^{Os}TP**, or **dU^{Os}TP**, respectively, produced similar peaks due to $Os^{2+/3+}$ at around 0.70 V

Table 2. Peak potentials of modified dNTPs and PEX products.

Conjugate nucleobase	A		G		U		C	
	dNTP	pex	dNTP	pex	dNTP	pex	dNTP	pex
$Os^{2+/3+}$[a]	0.75	–	0.73	0.70[c]	0.74	–	0.74	–
$Os^{2+/3+}$[b]	0.76	0.70	0.75	0.72	0.75	0.69	0.75	0.68
$Ru^{2+/3+}$[a]	1.21	–	1.20	–	1.16	–	1.16	–
$Ru^{2+/3+}$[b]	≈1.2	1.14[c]	1.21	–	1.18	1.13[c]	1.18	–

Apparent redox potential of the $M^{2+/3+}$ couples measured from the net square-wave voltammograms (V against Ag/AgCl/3 M KCl reference electrode), in situ SWV for 20 μM **dN^{Os}TP**s or 40 μM **dN^{Ru}TP**s conjugates, or ex situ SWV for PEX product (always containing one type of labeled nucleotide); [a] measured in 0.2 M NaCl/0.1 M Tris, pH 7.4; [b] measured in 0.2 M sodium acetate, pH 5.0; [c] pex^{noG} (separated extended strand; other data for pex^{rnd16} purified by Qiagen kit).

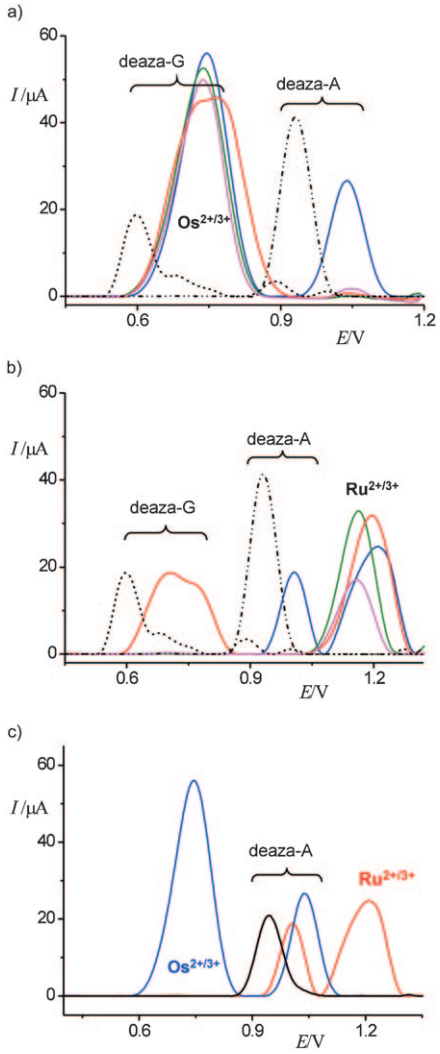


Figure 2. Square-wave voltammetric responses of labeled and unlabeled dNTPs. a) Baseline-corrected SWV voltammograms of **dA^{Os}TP** (blue), **dG^{Os}TP** (red), **dC^{Os}TP** (green), **dU^{Os}TP** (magenta), unlabeled 7-deaza-dATP (dash-dot-dot), and unlabeled 7-deaza-dGTP (dashed); all 20 µM. b) As in a) but with 40 µM **dN^{Ru}TP**s. c) Comparison of **dA^{Os}TP** (blue), **dA^{Ru}TP** (red), and 7-deaza-dATP (black). Background electrolyte, 0.2 M NaCl/0.1 M Tris at pH 7.4.

Table 3. Oxidation potentials of 7-deazapurine and purine moieties in dNTPs and PEX products.

	Unconjugated		Os		Ru	
	dNTP	pex	dNTP	pex	dNTP	pex
7-deaza-A[a]	0.93	–	1.04	–	1.01	–
7-deaza-A[b]	1.03	1.09	1.13	1.12	≈1.2	–
7-deaza-G[a]	0.60	–	0.77		0.71	–
7-deaza-G[b]	0.69	0.76[c]	≈0.8	≈0.8[c]	0.79	0.82[a]
A[a]	1.26	*nd*	–	–	–	–
A[b]	1.37	1.37	–	–	–	–
G[a]	0.95	1.00	–	–	–	–
G[b]	1.07	1.09	–	–	–	–

Potentials of net SWV peaks (V against Ag/AgCl/3 M KCl reference electrode); [a] measured in 0.2 M NaCl/0.1 M Tris, pH 7.4; [b] measured in 0.2 M sodium acetate, pH 5.0; in situ SWV for 20 µM unconjugated dNTPs and **dN^{Os}TP**s or 40 µM **dN^{Ru}TP**s; or ex situ SWV for PEX product pex^{noG} or [c] pex^{rnd16} (separated primer strand); nd: not detectable.

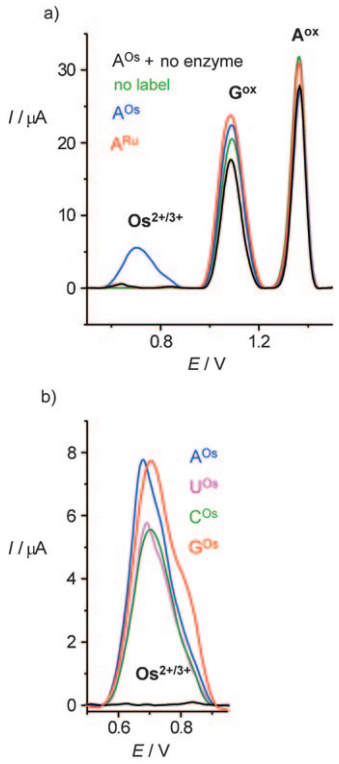


Figure 3. Electrochemical responses of Os- and Ru-labeled pex^{rnd16} products. a) Baseline-corrected ex situ SWV for pex^{rnd16}(**A^{Os}**) (blue), pex^{rnd16}-(**A^{Ru}**) (red), unlabeled pex^{rnd16} (green), and a negative PEX control (dNTP mix with **dA^{Os}TP** but no polymerase added, black); G^{ox} and A^{ox}, peaks due to oxidation of guanine and adenine, respectively. b) Detail of the Os$^{2+/3+}$ peak yielded by pex^{rnd16}(**A^{Os}**) (blue), pex^{rnd16}(**G^{Os}**) (red), pex^{rnd16}(**U^{Os}**) (magenta), pex^{rnd16}(**C^{Os}**) (green), and unlabeled pex^{rnd16} (black). Background electrolyte: 0.2 M sodium acetate, pH 5.0.

(Figure 3b). For pex^{rnd16}(**G^{Os}**), the osmium signal was accompanied by a shoulder at about 0.8 V, corresponding to electrochemical oxidation of the 7-deaza-G moiety (see above and Supporting Information).

In contrast to the well-developed signals due to the incorporated osmium-labeled nucleotides, no specific peak corresponding to Ru$^{2+/3+}$ was observed with the pex^{rnd16} products synthesized in the presence of either of the **dN^{Ru}TP**s (shown for pex^{rnd16}(**A^{Ru}**) in Figure 3a). Luminescence measurements (see below) indicated that the absence of a specific Ru$^{2+/3+}$ electrochemical signal was not caused by an absence of the Ru labels in the PEX products, but probably by overlap of the peak with one of the two oxidation peaks of purines, which were rather abundant in the double-strand pex^{rnd16} (19 guanines and 12 adenines for each of the four **A^{Ru}** incorporated). A change of the background electrolyte to NaCl/Tris at pH 7.4 (as used for the dNTPs in Figure 2) did not result in unmasking of the Ru$^{2+/3+}$ signal in the pex^{rnd16}(**N^{Ru}**) product and, moreover, it was not possible to detect the adenine oxidation signal in the latter medium due to a negatively shifted background discharge. To enable electrochemical detection of the incorporated Ru tags, we therefore designed primers and templates to prepare PEX products lacking

either of the natural purine residues in the extended primer strand. Using the primer prim^{noG} and 5′-biotinylated template temp^{noG} (see Table 1), products pex^{noG}(**A**^{M}) and pex^{noG}(**U**^{M}) were prepared, and the labeled strands were isolated using a procedure involving streptavidin-coated magnetic beads described in our previous work.[16,17,22] The resulting single-strand ONs were analyzed by ex situ SWV as described above. Unlabeled pex^{noG} yielded only peak A^{ox}, in agreement with the absence of G, any other purine derivative, or any metal-containing tag (Figure 4a). In addition to the peak A^{ox}, pex^{noG}(**A**^{Ru}) produced another signal at 1.14 V, that is, close to the potential of the guanine peak G^{ox}. Since no G was present in the PEX product, the latter peak was attributed to the incorporated **A**^{Ru} conjugate. However, when the **dA**^{Ru}**TP** conjugate was replaced in the PEX reaction mixture with unsubstituted 7-deaza-dATP, the resulting products yielded an oxidation peak at around 1.1 V (i.e., close to the Ru$^{2+/3+}$ signal; Table 3). A similar peak was detected for pex^{noG}(**A**^{Os}). A comparison with the electrochemical responses of the dNTPs suggested that the 7-deaza-A moiety was responsible for the signal produced by the Ru-free PEX products containing 7-deaza-A derivatives. Analysis of the "forward" and "backward" components[24] of the square-wave voltammogram (Figure 4c) revealed the reversible component of the signal yielded by pex^{noG}(**A**^{Ru}) at 1.14 V (showing a negative, i.e. reduction, peak on the backward curve), thus confirming its assignment to the Ru$^{2+/3+}$ redox couple. For the Ru-free PEX products, the peak attributed to oxidation of 7-deaza-A was irreversible as expected (shown in Figure 4c for pex^{noG}(**A**^{Os}), which, on the other hand, yielded a reversible signal due to the Os$^{2+/3+}$ redox couple).

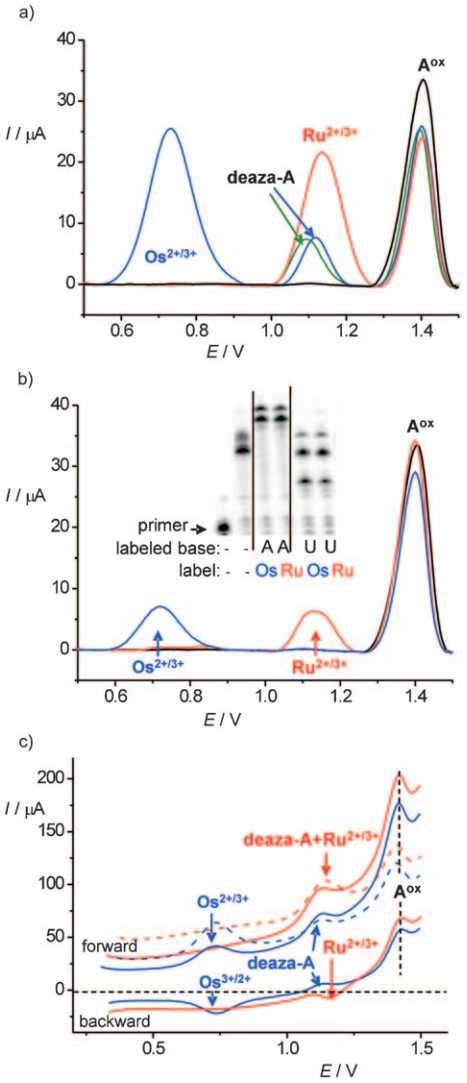


Figure 4. Electrochemical responses of single-strand pex^{noG} products lacking guanine residues. a) Baseline-corrected SWV for pex^{noG}(**A**^{Os}) (blue), pex^{noG}(**A**^{Ru}) (red), pex^{noG} with unlabeled 7-deaza-A (green), and unlabeled pex^{noG} containing only natural purines (black). b) pex^{noG}(**U**^{Os}) (blue), pex^{noG}(**U**^{Ru}) (red), and unlabeled pex^{noG} (black). c) The "forward" and "backward" components of SWV for pex^{noG}(**A**^{Os}) (blue) and pex^{noG}-(**A**^{Ru}) (red); dashed curves are the corresponding net voltammograms. Other conditions as in Figure 3a, b. Inset in b): PAGE of the pex^{noG}(**A**^{M}) and pex^{noG}(**U**^{M}) products.

In pex^{noG}(**U**^{M}), no 7-deaza-A was present and thus no signal corresponding to this base could be detected. Both pex^{noG}(**U**^{Os}) and pex^{noG}(**U**^{Ru}) produced (in addition to peak A^{ox} due to adenine) only the reversible metal-specific signals (Figure 4b). Lower intensities of the Os and Ru peaks observed with the pex^{noG}(**U**^{M}) products were in accord with early termination of DNA polymerization on the temp^{noG} template with **dU**^{M}**TP** (see the inset in Figure 4b), resulting in incorporation of fewer tags per ON molecule. Such "side-by-side" incorporation of **U**^{M} was required on the temp^{noG} template involving A$_n$ blocks (see Table 1), while **A**^{M} was easily introduced at four isolated positions and full-length products were obtained.

Photophysical properties of the Os- and Ru-labeled dNTPs and PEX products: In order to explore the possibilities of using optical methods in analysis of the Ru- or Os-labeled nucleic acids, we studied the photophysical properties of the **dN**^{M}**TP**s as well as those of the modified PEX products. The UV/Vis absorption spectra of the **dN**^{M}**TP**s displayed three distinct bands: close to 290 nm, at ≈345–350 nm (for pyrimidine conjugates) or ≈360–375 nm (for 7-deazapurine conjugates), and close to 450 nm (see, for example, Figure 5a; more details are given in the Supporting Information). Comparison with the spectrum of [Ru(bpy)$_3$]$^{2+}$ suggests that the bands at 290 nm and 450 nm may be ascribed to the metal–bipyridine complexes,[19] specifically to a π→π* transition within the bpy moieties and to a d→π* transition within the Ru complex, respectively. Upon excitation within the absorption band at 450 nm, all of the **dN**^{Ru}**TP**s (but none of the **dN**^{Os}**TP**s) exhibited strong red luminescence with emission maxima at around 640 nm (Table 4, Figure 5a), in accordance with our previous observations on the corresponding Ru-modified nucleosides.[19] When recording the emission spectra under exclusion of air (under argon), the emissions of the Ru complexes were slightly stronger, while the Os complexes remained devoid of any measurable lumines-

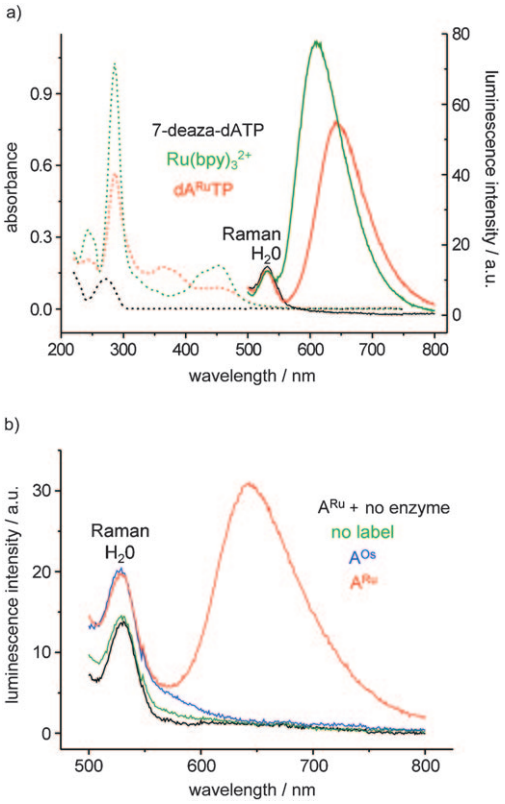


Figure 5. a) UV/Vis absorption (dotted) and emission luminescence (solid) spectra of 7-deaza-dATP (black), **dA^{Ru}TP** (red), and $[Ru(bpy)_3]^{2+}$ (green). Concentrations 100 μM for UV/Vis or 200 nM for luminescence, diluted in water. b) Luminescence spectra of pex^{rnd16} products: pex^{rnd16}-(**A^{Ru}**) (red), pex^{rnd16}(**A^{Os}**) (blue), unlabeled pex^{rnd16} (green), and a negative PEX control (dNTP mix with **dA^{Os}TP** but no polymerase added; black). The PEX products were prepared as described in the caption to Figure 3. For all luminescence spectra, the excitation wavelength was 450 nm.

cence (up to 5 μM concentrations). The luminescence of the $Ru(bpy)_3$ complexes was retained in the Ru-labeled PEX products (Table 4, Figure 5b).

Quantum yields obtained for the PEX products were in general lower than those exhibited by the **dN^{Ru}TP**s, suggesting a certain degree of luminescence quenching in the rather complicated environment provided by the DNA molecule. The quenching was even more pronounced in the pex^{rnd16} products incorporating four labeled pyrimidines, which exhibited considerably lower Φ, as compared to the corresponding pex^{rnd16} bearing labeled 7-deazapurines (Table 4). Since no sign of light emission at around 650 nm was detected for the Os-labeled or unlabeled PEX products as well as for the negative PEX control (Figure 5b), analysis of the luminescence proved useful for monitoring the incorporation of the Ru-conjugated nucleotides and distinguishing the Ru-labeled nucleic acids from those bearing different types of labels. Using the luminescence, the **dN^{Ru}TP**s could be detected at 10^{-8} M concentrations, that is to say, the detection limits were in the same range as attained for the **dN^{Os}TP**s using voltammetry (see Supporting Information).

Electrochemical multicolor DNA coding using base-modified dNTP conjugates: The efficient methodology of modified dNTP synthesis through aqueous cross-coupling reactions[14] opens up the possibility of developing a broad palette of redox (or other) DNA labels applicable in "multicolor" (multipotential) DNA labeling. Using different electrochemically oxidizable or reducible tags differing in their redox potentials, DNA molecules encoded by specific electrochemical signals can easily be constructed. Taking into consideration the problematic parallel incorporations of Os and Ru tags and the fact that the oxidation signal of Ru is eclipsed by oxidation of G (see above), we chose the **dN^{Os}TP**s in combination with other previously reported redox-active labels[15,16] for multicolor DNA labeling. For example, SWV responses of the pex^{rnd16} products bearing diverse electroactive labels are shown in Figure 6a. Each PEX product was synthesized with a particular dNTP mix involving one specifically labeled dNTP (**dA^{Os}TP**, **dU^{PhNO2}TP**, **dC^{PhNH2}TP**,[17] or **dG^{Fc}TP**) complemented by the remaining three natural dNTPs. The resulting electrochemical signals corresponded to the labeled dNTP used, thus indicating the presence of the particular label in a given PEX product (Figure 6a). In addition to the diverse peak potentials, the character of the electrode process (irreversible reduction or oxidation, reversible redox) can also be utilized for a precise discrimination among different labels. In particular, a look at the reversibility of oxidation appears to be useful for discrimination of the 7-deaza-G peak (produced by all of the tested 7-substituted derivatives of this base) that interferes with the $Os^{2+/3+}$ signal. Note also that the relative intensities of the individual signals corresponded well to the number of electrons involved in the apparent electrode processes, i.e., the irreversible one-electron oxidation of $PhNH_2$, the reversible one-electron redox of the Fc and Os tags giving a sum of two electrons in the net SWV signal,[22] and the irreversible four-electron reduction of $PhNO_2$.

Table 4. Luminescence maxima and quantum yields for **dN^{Ru}TP**s and Ru-labeled PEX products.

	dN^{Ru}TP		pex^{N}[a]		pex^{rnd16}(**N^{Ru}**)[b]	
	Emission [nm]	Φ	Emission [nm]	Φ	Emission [nm]	Φ
A^{Ru}	642	0.0187	642	0.0120	642	0.0133
C^{Ru}	646	0.0132	650	0.0089	648	0.0031
U^{Ru}	645	0.0156	643	0.0133	650	0.0034
dG^{Ru}	635	0.0111	639	–	638	0.0146
$[Ru(bpy)_3]^{2+}$[c]	615	0.0348	–	–	–	–

[a] pex^{A}, pex^{C}, or pex^{U} bearing a single Ru label (synthesized with templates temp^{A}, temp^{C}, or temp^{U} using **dA^{Ru}TP**, **dC^{Ru}TP**, or **dU^{Ru}TP**, respectively, always complemented with dGTP); [b] pex^{rnd16}(**N^{Ru}**) bearing four Ru labels per molecule (**dN^{Ru}TP** + 3 other natural dNTPs as in Figure 1); [c] unconjugated $[Ru(bpy)_3]^{2+}$. Luminescence spectra measured in 3.3 mM Tris-Cl at pH 8.5; excitation wavelength 450 nm.

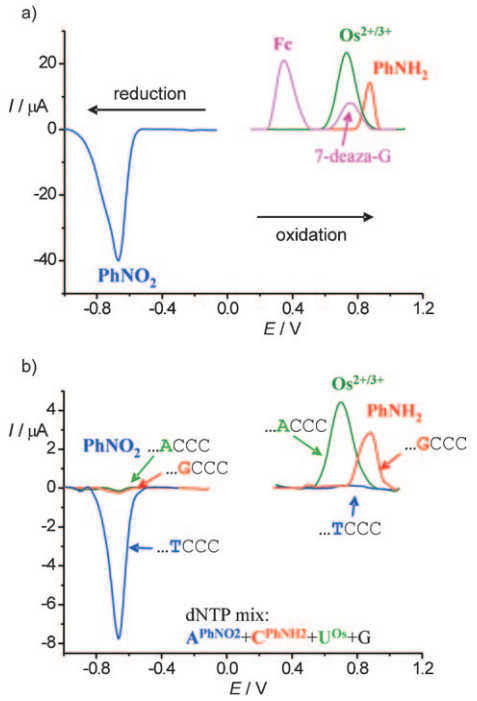
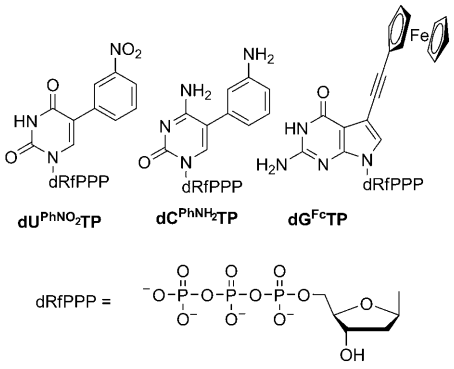


Figure 6. Electrochemical "multicolor" DNA labeling using dNTP conjugates and SNP detection. a) SWV responses for pex^{rnd16}(**A^{Os}**) (green), pex^{rnd16}(**G^{Fc}**) (magenta), pex^{rnd16}(**U^{PhNO2}**) (blue), and pex^{rnd16}(**C^{PhNH2}**) (red). b) PEX probing of a single nucleotide polymorphism using a mix of **dU^{Os}TP**, **dC^{PhNH2}TP**, and **dA^{PhNO2}TP** complemented with unlabeled dGTP. The PEX reactions were conducted with templates temp^A, temp^C, or temp^U (sequences complementary to the synthesized stretches shown in the figure). Conditions as in Figure 3a, b.



Oligonucleotides labeled with diverse redox tags have been applied as signaling probes in parallel DNA hybridization assays.[3,22,25] However, the DNA polymerase-based DNA labeling approach using redox-active dNTP conjugates offers more possibilities in electrochemical DNA sensing. Sequence-specific incorporation of a specific nucleobase conjugate can easily be used for single nucleotide polymorphism (SNP) probing (Figure 6b). Here, we used model ON templates temp^A, temp^T, and temp^C (Table 1) for probing the T/A/G SNP. The dNTP mix used for all PEX reactions contained labeled **dU^{Os}TP**, **dA^{PhNO2}TP**, **dC^{PhNH2}TP**, and natural dGTP. The electrochemical responses of the resulting products perfectly matched the complementarity between the dNTP incorporated (as indicated by the specific SWV signal) and the nucleobase within the SNP site of the given template: for temp^A, temp^T, and temp^C, well-defined peaks due to $Os^{2+/3+}$, $PhNO_2$, and $PhNH_2$, respectively, were detected.

Conclusion

All eight combinations of [Ru(bpy)$_3$]$^{2+}$ and [Os(bpy)$_3$]$^{2+}$ complexes of all four nucleobase (U, C, 7-deaza-A, 7-deaza-G) nucleoside triphosphates have been prepared in a single step by aqueous Sonogashira cross-couplings of the corresponding halogenated dNTPs with terminal acetylene derivatives of the complexes. Despite the very bulky, inorganic, and charged nature of the complexes, the corresponding modified dNTPs proved to be good substrates for Vent (exo-) and Pwo polymerases and could be efficiently incorporated into diverse sequences of DNA by primer extension. The only limitation was the incorporation of two labeled dNTPs at adjacent positions, which usually caused termination of the chain.

Square-wave voltammetry has been used for electrochemical detection of the labeled nucleic acids based on the reversible oxidations $Ru^{2+/3+}$ or $Os^{2+/3+}$. The redox potentials of the Ru^{2+} complexes (1.1–1.25 V) were observed to be very close to the potential for G oxidation (1.1 V), and in DNA the signal of Ru^{2+} was usually invisible due to overlap with the strong G oxidation signal. On the other hand, the potentials of Os^{2+} complexes (0.75 V) differ significantly, thereby allowing specific DNA labeling. Compared to previously studied ferrocenylethynyl, aminophenyl, and nitrophenyl tags, the redox potentials of the Os complexes were not found to be significantly dependent on the nucleobase to which they were attached and/or did not respond to incorporation into DNA. The Ru^{2+} complexes were oxidized at higher potentials than the deazapurine part, which is another reason why these complexes are less suitable for redox labeling of DNA to be used in bioanalysis. On the other hand, Ru^{2+}-labeled ONs (unlike the corresponding Os complexes) are luminescent. Therefore, it can be concluded that Os-labeling of DNA is useful for electrochemical detection, while Ru-labeling can be used for independent detection by luminescence spectroscopy. The Os-labeled dATP, in combination with previously reported dNTPs bearing ferrocene, aminophenyl, and nitrophenyl tags, has been successfully used for "multicolor" redox labeling of DNA and, to the best of our knowledge, we have presented the first example of a complete set of four different independent redox labels for all four nucleobases in DNA. The multicolor coding has also been successfully used for SNP typing.

Experimental Section

General chemistry: All cross-coupling reactions were performed under argon atmosphere. Et_3N was degassed in vacuo and stored over molecular sieves under argon. Compounds 5-I-dUTP (ref. [20]), 5-I-dCTP (ref. [17]), 7-I-7-deaza-dATP (ref. [15]), 7-I-7-deaza-dGTP (ref. [21]), [Os(bpy)$_2$Cl$_2$] (ref. [26]), and **1^{Ru}** (ref. [10]) were prepared according to the literature procedures. Other chemicals were purchased from commercial suppliers and were used as received. NMR spectra were recorded on Bruker Avance II 600 MHz (600 MHz for ^{1}H and 151 MHz for ^{13}C) or Bruker Avance 500 (500 MHz for ^{1}H and 125.8 MHz for ^{13}C) spectrometers from sample solutions in D_2O, [D_3]acetonitrile, or [D_6]acetone. Chemical shifts (in ppm, δ scale) were referenced to the solvent signal (D_2O: referenced to dioxane 3.75 ppm, pH 7.1, for ^{1}H NMR and to H_3PO_4 0.00 ppm for ^{31}P NMR; [D_6]acetone: 2.05 ppm for ^{1}H NMR and 29.8 ppm (CD_3 group of [D_6]acetone) for ^{13}C NMR). Coupling constants (J) are given in Hz. NMR spectra of dNTPs were measured in phosphate buffer at pH 7.1.

Compound 1Os: Ethylene glycol (50 mL) was added to a mixture of 5-ethynyl-2,2′-bipyridine (180 mg, 1 mmol) and [Os(bpy)$_2$Cl$_2$] (574 mg, 1 mmol, 1 equiv) and the mixture was stirred for 2 h at 150 °C. The solvent was then evaporated under reduced pressure. The residue was dissolved in water and then a saturated solution of NH_4PF_6 was added. The precipitate that formed was collected by filtration. The crude product was purified by column chromatography on silica gel using a mixture of CH_3CN/H_2O/saturated aqueous KNO_3 (10:1:0.1) as eluent. The appropriate fractions were combined and the solvent was removed in vacuo. The residue was dissolved in a small amount of EtOH and the KNO_3 was filtered off. The product was dissolved in water and a saturated solution of NH_4PF_6 was added. The product precipitated from the solution as the hexafluorophosphate salt. The precipitate was collected by filtration to give a dark black-green solid (522 mg, 54 %). ^{1}H NMR (600 MHz, [D_6]acetone): δ = 4.11 (s, 1H; HC≡C), 7.46–7.52 (m, 5H; H-5″ and 4×H-5-bpy), 7.92 and 7.94 (2×ddd, 2×1H, $J_{6,5}$ = 5.7, $J_{6,4}$ = 1.4, $J_{6,3}$ = 0.8 Hz; H-6-bpy), 7.97–8.05 (m, 9H; H-4′, H-4″, H-6′, H-6″, 4×H-4-bpy and H-6-bpy), 8.08 (ddd, 1H, $J_{6,5}$ = 5.7, $J_{6,4}$ = 1.4, $J_{6,3}$ = 0.8 Hz; H-6-bpy), 8.78–8.82 ppm (m, 6H; H-3′, H-3″, 4×H-3-bpy); ^{13}C NMR (151 MHz, [D_6]acetone): δ = 78.64 (C≡CH), 87.08 (HC≡C), 124.19 (C-5′), 124.98, 125.45, 125.47, 125.51, 125.60, and 126.13 (CH-3′, CH-3″, and CH-3-bpy), 129.13, 129.16, 129.20, 129.21, and 129.47 (CH-5″ and CH-5-bpy), 138.22, 138.37, 138.39 and 138.43 (CH-4″ and CH-4-bpy), 140.62 (CH-4′), 151.60, 151.88, 151.94, and 152.23 (CH-6″ and CH-6-bpy), 153.94 (CH-6′), 159.37, 159.82, 159.85, 159.97, and 160.18 ppm (C-2′, C-2″, and C-2-bpy); ESI MS: m/z (%): 829 (100) [M^{2+}+PF_6^-], 683 (35) [M^{2+}−2PF_6^-]. IR: $\tilde{\nu}$ = 3280, 3122, 2118, 1607, 1448, 840 cm^{-1}.

General procedure for the Sonogashira cross-coupling reaction of [M(bpy)$_2$(5-ethynyl-2,2′-bpy)]$^{2+}$(PF_6^-)$_2$ (M = Ru or Os) with halogenated nucleoside triphosphates: A 2:1 mixture of H_2O/CH_3CN (1 mL) followed by Et_3N (70 μL, 10 equiv) were added to an argon-purged flask containing halogenated nucleoside triphosphate 7-I-7-deaza-dATP, 7-I-7-deaza-dGTP, 5-I-dCTP, or 5-I-dUTP (0.05 mmol), [M(bpy)$_2$(5-ethynyl-2,2′-bpy)]$^{2+}$(PF_6^-)$_2$ (where M is either Ru or Os) (0.075 mmol, 1.5 equiv), and CuI (1 mg, 10 mol %). In a separate flask, $Pd(OAc)_2$ (0.6 mg, 0.0025 mmol, 5 mol %) and $P(C_6H_4SO_3Na)_3$ (7.1 mg, 0.0125 mmol, 5 equiv with respect to Pd) were combined, the flask was evacuated and purged with argon, and then a 2:1 mixture of H_2O/CH_3CN (0.5 mL) was added. This catalyst solution was injected into the reaction mixture, which was then stirred at 65–70 °C for 1 h. Thereafter, the solvent was evaporated in vacuo. The products were purified by RP-HPLC using a linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H_2O to 0.1 M TEAB in H_2O/MeOH (1:1) as eluent. The products were isolated after freeze-drying as red powders (Ru complexes and Fc complex) or as dark black-green powders (Os complexes) (isolated as 3×Et_3N salts).

dU^{Ru}TP: The product was isolated as a red powder (10 mg, 12 %; 1:1 mixture of diastereoisomers, Et_3NH^+ salt). ^{1}H NMR (500 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pH 7.1): δ = 1.27 (t, 36H, J_{vic} = 7.4 Hz; CH_3CH_2N), 2.33 and 2.40 (2×brm, 2×2H; H-2′), 3.19 (q, 24H, J_{vic} = 7.4 Hz; CH_3CH_2N), 4.14–4.28 (brm, 6H; H-4′ and H-5′), 4.60 (brm, 2H; H-3′), 6.24 and 6.26 (2×t, 2×1H, $J_{1',2'}$ = 6.7 Hz; H-1′), 7.31–7.41, 7.76–7.82, 7.85–7.89, 7.98–8.11, 8.22–8.29, and 8.48–8.59 ppm (5×m, 48H; H-6 + H-bpy); ^{31}P NMR (^{1}H-decoupled) (202.3 MHz, D_2O, ref$_{H3PO4}$ = 0 ppm, pH 7.1): δ = −19.26 (br, P_β), −10.15 (br, P_α), −5.07 ppm (br, P_γ); ESI MS: m/z (%): 1057.0 (100) [M^+−2PF_6^-−2H].

dU^{Os}TP: The product was isolated as a black-green powder (15 mg, 17 %; 1:1 mixture of diastereoisomers, Et_3NH^+ salt). ^{1}H NMR (500 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pH 7.1): δ = 1.27 (t, 18H, J_{vic} = 7.3 Hz; CH_3CH_2N), 2.34 and 2.40 (2×brm, 2×2H; H-2′), 3.19 (q, 12H, J_{vic} = 7.3 Hz; CH_3CH_2N), 4.14–4.28 (brm, 6H; H-4′ and H-5′), 4.65 (brm, 2H; H-3′), 6.25 and 6.26 (2×t, 2×1H, $J_{1',2'}$ = 6.4 Hz; H-1′), 7.20–7.33, 7.65–7.74, 7.75–7.95, and 8.03–8.11 (4×m, 36H; H-bpy), 8.27 (s, 2H; H-6), 8.45–8.58 ppm (m, 10H; H-bpy); ^{31}P NMR (^{1}H-decoupled) (202.3 MHz, D_2O, ref$_{H_3PO_4}$ = 0 ppm, pH 7.1): δ = −21.70 (br, P_β), −10.75 (d, J = 19.6 Hz, P_α), −7.11 ppm (brd, J = 18.3 Hz, P_γ); ESI MS: m/z (%): 1152.3 (55) [M^+ −2PF_6^-+2H].

dC^{Ru}TP: The product was isolated as a red powder (29 mg, 35 %; 1:1 mixture of diastereoisomers, Et_3NH^+ salt). ^{1}H NMR (500 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pH 7.1): δ = 1.27 (t, 18H, J_{vic} = 7.3 Hz; CH_3CH_2N), 2.12 and 2.35 (2×brm, 2×2H; H-2′), 3.20 (q, 12H, J_{vic} = 7.3 Hz; CH_3CH_2N), 4.04–4.24 (brm, 6H; H-4′ and H-5′), 4.53 (brm, 2H; H-3′), 6.04 and 6.10 (2×t, 2×1H, $J_{1',2'}$ = 6.5 Hz; H-1′), 7.29–7.44, 7.72–7.90, 7.95–8.21, 8.24–8.35, and 8.47–8.65 ppm (5×m, 48H; H-6 + H-bpy); ^{31}P NMR (^{1}H-decoupled) (202.3 MHz, D_2O, ref$_{H_3PO_4}$ = 0 ppm, pH 7.1): δ = −21.36 (br, P_β), −10.64 (d, J = 17.0 Hz, P_α), −7.30 (br, P_γ); ESI MS: m/z (%): 1055.7 (100) [M^+−2PF_6^-−3H].

dC^{Os}TP: The product was isolated as a black-green powder (15 mg, 17 %; 1:1 mixture of diastereoisomers, Et_3NH^+ salt). ^{1}H NMR (500 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pH 7.1): δ = 1.27 (t, 18H, J_{vic} = 7.3 Hz; CH_3CH_2N), 2.19 and 2.39 (2×brm, 2×2H; H-2′), 3.20 (q, 12H, J_{vic} = 7.3 Hz; CH_3CH_2N), 4.04–4.28 (brm, 6H; H-4′ and H-5′), 4.57 (brm, 2H; H-3′), 6.13 and 6.17 (2×brm, 2×1H; H-1′), 7.19–7.38, 7.61–7.98, 8.01–8.23, and 8.43–8.66 ppm (4×m, 48H; H-6 + H-bpy); ^{31}P NMR (^{1}H-decoupled) (202.3 MHz, D_2O, ref$_{H_3PO_4}$ = 0 ppm, pH 7.1): δ = −21.60 (br, P_β), −10.68 (br, P_α), −7.12 ppm (br, P_γ); ESI MS: m/z (%): 1146.1 (100) [M^+ −2PF_6^-−2H].

dA^{Ru}TP: The product was isolated as a red powder (18 mg, 21 %; 1:1 mixture of diastereoisomers, Et_3NH^+ salt). ^{1}H NMR (500 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pH 7.1): δ = 1.27 (t, 36H, J_{vic} = 7.3 Hz; CH_3CH_2N), 1.90–2.21 (brm, 4H; H-2′), 3.19 (q, 24H, J_{vic} = 7.3 Hz; CH_3CH_2N), 3.96–4.25 (brm, 6H; H-4′ and H-5′), 4.36 and 4.53 (2×brm, 2×1H; H-3′), 6.00 (brm, 2H; H-1′), 6.60–8.90 ppm (m, 50H; H-2,6 + H-bpy); ^{31}P NMR (^{1}H-decoupled) (202.3 MHz, D_2O, ref$_{H_3PO_4}$ = 0 ppm, pH 7.1): δ = −19.49 (br, P_β), −10.12 (br, P_α), −5.62 ppm (br, P_γ); ESI MS: m/z (%): 1001.0 (75) [M^+−2PF_6^-−PO_3^{2-}], 1080.9 (75) [M^+−2PF_6^-].

dA^{Os}TP: The product was isolated as a black-green powder (24 mg, 26 %; 1:1 mixture of diastereoisomers, Et_3NH^+ salt). ^{1}H NMR (500 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pH 7.1): δ = 1.27 (t, 36H, J_{vic} = 7.3 Hz; CH_3CH_2N), 2.02, 2.13, and 2.20 (3×brm, 4H; H-2′), 3.19 (q, 24H, J_{vic} = 7.3 Hz; CH_3CH_2N), 3.85, 4.00, 4.05, and 4.14 (4×brm, 6H; H-4′ and H-5′), 4.42 and 4.53 (2×brm, 2×1H; H-3′), 6.03 and 6.05 (2×brm, 2×1H; H-1′), 6.78, 6.95, 7.09–7.38, 7.41–8.19, and 8.36–8.80 ppm (5×brm, 50H; H-2,6 + H-bpy); ^{31}P NMR (^{1}H-decoupled) (202.3 MHz, D_2O, ref$_{H_3PO_4}$ = 0 ppm, pH 7.1): δ = −21.64 (br, P_β), −10.55 and −10.43 (2×d, J = 21.0 Hz, P_α), −5.62 ppm (brd, J = 18.0 Hz, P_γ); ESI MS: m/z (%): 1171.0 (100) [M^+ −2PF_6^-], 1091.0 (50) [M^+−2PF_6^-−PO_3^{2-}].

dG^{Ru}TP: The product was isolated as a red powder (17 mg, 20 %; 1:1 mixture of diastereoisomers, Et_3NH^+ salt). ^{1}H NMR (500 MHz, D_2O, ref$_{dioxane}$ = 3.75 ppm, pH 7.1): δ = 1.27 (t, 18H, J_{vic} = 7.3 Hz; CH_3CH_2N), 1.90–2.21 (brm, 4H; H-2′), 3.19 (q, 12H, J_{vic} = 7.3 Hz, CH_3CH_2N), 3.91–4.20 (brm, 6H; H-4′ and H-5′), 4.46 and 4.54 (2×brm, 2×1H; H-3′), 5.98–6.15 (brm, 2H; H-1′), 6.78–7.05, 7.24–7.45, 7.55–8.20, and 8.28–8.69 ppm (4×m, 48H; H-6 + H-bpy); ^{31}P NMR (^{1}H-decoupled) (202.3 MHz, D_2O, ref$_{H_3PO_4}$ = 0 ppm, pH 7.1): δ = −21.23 (br, P_β), −10.38 (br, P_α), −6.49 ppm (br, P_γ); ESI MS: m/z (%): 1096.7 [M^+−2PF_6^-−2H], 1243.7 (50) [M^+−PF_6^-].

dG^{Os}TP: The product was isolated as a black-green powder (25 mg, (28 %; 1:1 mixture of diastereoisomers, Et_3NH^+ salt). ^{1}H NMR

(500 MHz, D_2O, $ref_{dioxane}=3.75$ ppm, pH 7.1): $\delta=1.27$ (t, 18H, $J_{vic}=7.3$ Hz; CH_3CH_2N), 2.06–2.46 (br m, 4H; H-2′), 3.19 (q, 12H, $J_{vic}=7.3$ Hz; CH_3CH_2N), 3.94–4.25 (br m, 6H; H-4′ and H-5′), 4.48–4.70 (br m, 2H; H-3′), 6.14–6.32 (br m, 2H; H-1′), 6.98–7.41, 7.54–8.13, and 8.32–8.73 ppm (3×m, 48H; H-6 + H-bpy); ^{31}P NMR (^1H-decoupled) (202.3 MHz, D_2O, $ref_{H3PO4}=0$ ppm, pH 7.1): $\delta=-20.71$ (br, P_β), -10.15 (br, P_α), -6.05 ppm (br, P_γ); ESI MS: m/z (%): 1186.9 (100) [$M^+-2PF_6^-$].

dG^{Fc}TP: This compound was prepared analogously to the General Procedure using 7-I-7-deaza-dGTP (0.05 mmol) and ethynylferrocene (0.075 mmol, 1.5 equiv). The product was isolated as a red powder (14 mg, 27%). ^1H NMR (500 MHz, D_2O, pH 7.1, phosphate buffer, $ref_{dioxane}=3.75$ ppm): $\delta=1.27$ (br s, 32H; CH_3CH_2N), 2.41 (ddd, 1H, $J_{gem}=14.1$, $J_{2'b,1'}=6.2$, $J_{2'b,3'}=3.1$ Hz; H-2′b), 2.66 (ddd, 1H, $J_{gem}=14.1$, $J_{2'a,1'}=8.1$, $J_{2'a,3'}=6.3$ Hz; H-2′a), 3.19 (br s, 24H; CH_3CH_2N), 4.15 (br m, 2H; H-5′), 4.21 (br m, 1H; H-4′), 4.37 (br s, 5H; cp), 4.59 (br s, 2H; cp), 4.72 (br m, 1H; H-3′), 4.83 (br s, 2H; cp), 6.41 (dd, 1H, $J_{1'2'}=8.1$, 6.2 Hz; H-1′), 7.32 ppm (s, 1H; H-6); ^{13}C NMR (151 MHz, D_2O, pH 7.1, $ref_{dioxane}=69.3$ ppm): $\delta=10.95$ (CH_3CH_2N), 40.89 (CH_2-2′), 49.34 (CH_3CH_2N), 61.70 (C-cp), 68.31 (d, $J_{C,P}=6$ Hz; CH_2-5′), 71.99, 72.77, and 73.80 (CH-cp), 74.12 (CH-3′), 81.11 (C≡C-fer), 85.76 (CH-1′), 87.85 (d, $J_{C,P}=8$ Hz, CH-4′), 92.94 (C≡C-fer), 102.27 and 102.88 (C-4a,5), 125.54 (CH-6), 153.56 (C-7a), 156.08 and 163.63 ppm (C-2,4); ^{31}P NMR (^1H-decoupled) (202.3 MHz, D_2O, pH 7.1, $ref_{H3PO4}=0$ ppm): $\delta=-21.22$ (t, $J=19.4$ Hz, P_β), -10.23 (d, $J=19.4$ Hz; P_α), -6.20 ppm (d, $J=19.4$ Hz; P_γ); ESI MS: m/z (%): 733.6 (100) [M^++Na], 654.8 (40) [$M^+-PO_3^{2-}$].

Biochemical materials: Synthetic ONs were purchased from VBC genomics (Austria); for their sequences, see Table 1. Dynabeads M-280 streptavidin (DB_{Stv}) were obtained from Dynal A.S. (Norway); Pwo DNA polymerase from PEQLAB (Germany); Vent (exo-) DNA polymerase, Klenow (exo-) fragment, and T4 polynucleotide kinase from New England Biolabs (Great Britain); DyNAzyme II DNA polymerase from Finnzymes (Finland); standard nucleoside triphosphates (dATP, dTTP, dCTP, and dGTP) from Sigma; 7-deaza-dGTP and 7-deaza-dATP from Jena Bioscience (Germany), and γ-[^{32}P]-ATP from MP Empowered Discovery (USA). Other chemicals were of analytical grade.

Primer extension: A primer (0.7 μM) was mixed with a template ON (0.7 μM), dNTPs (125 μM each), and a DNA polymerase (0.2–0.5 U per sample). Reaction mixtures were incubated for 30 min at 37 °C (Klenow (exo-) polymerase) or at 60 °C (thermostable polymerases). For polyacrylamide gel electrophoresis (PAGE) experiments, the primer was ^{32}P-labeled at its 5′-end. For electrochemical and luminescence analysis, the PEX products were either purified using Qiagen Nucleotide Removal kit, or the extended primer strand was separated using magnetic beads.

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} bromophenol blue) and subjected to electrophoresis in 15% denaturing polyacrylamide gel containing 1× TBE buffer (pH 8) and 7 M urea at 25 W for 50 min. Gels were dried, autoradiographed, and visualized using a phosphorimager.

Magnetic separation of PEX product strands: A protocol described in detail in our previous reports[15,16] was used. Briefly, the PEX products were captured at DB_{Stv} through biotin tags tethered to the 5′-ends of the template strands. Aliquots (50 μL) of the PEX reaction mixtures were added to the DB_{stv} (25 μL of the stock suspension was washed with 0.3 M NaCl/10 mM Tris-HCl at pH 7.4 (buffer H; 2×100 μL)). After shaking for 30 min at 20 °C, the beads were washed with PBS (0.14 M NaCl, 3 mM KCl, 4 mM Na_3PO_4, pH 7.4) containing 0.01% Tween 20 (3×100 μL) and with buffer H (3×100 μL), and then resuspended in deionized water (50 μL). The extended primer strands were released by heating at 65 °C for 2 min. Each medium exchange was performed using a magnetoseparator (Dynal, Norway). Prior to the ex situ electrochemical measurements, NaCl was added to the samples (final concentration 0.2 M).

Electrochemical analysis: The PEX products were analyzed by means of ex situ (adsorptive transfer stripping) square-wave voltammetry (SWV). The PEX products were accumulated over a period of 60 s from 5 μL aliquots containing 0.2 M NaCl on the surface of a basal-plane pyrolytic graphite electrode (PGE; prepared and pre-treated as described previously[27]). The electrode was then rinsed with deionized water and placed in the electrochemical cell. Electrochemical responses of the labeled and unlabeled dNTPs (Figure 2) were measured in situ (with the analyte dissolved in the background electrolyte). Settings for measurements of the oxidation responses of $Os^{2+/3+}$, $Ru^{2+/3+}$, Fc, $PhNH_2$, purine, and 7-deaza-purine residues were as follows: initial potential −1.0 V, final potential +1.5 V, pulse amplitude 25 mV, frequency 200 Hz, potential step 5 mV. Settings for measurements of the reduction of the $PhNO_2$ tag were as follows: initial potential 0 V, final potential −1.5 V, other parameters the same as above). The measurements were performed at ambient temperature in 0.2 M sodium acetate at pH 5.0 or in 0.2 M NaCl/0.1 M Tris at pH 7.4 using an Autolab analyzer (EcoChemie, The Netherlands) in a three-electrode set-up (with the PGE as working, Ag/AgCl/3 M KCl as reference, and platinum wire as counter electrode). The voltammograms were baseline-corrected by means of a moving average algorithm (GPES 4 software, EcoChemie).

Absorption UV/Vis spectra: UV/Vis spectra were measured in the range 220–750 nm on a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA), in water for dNTP or in 3.3 mM Tris at pH 8.5 for the PEX products.

Emission luminescence spectra: These were measured in the range 500–800 nm on a PC1 spectrofluorimeter (ISS, USA) in 0.1 M NaCl/3.3 mM Tris at pH 8.5 using an excitation wavelength of 450 nm and band-passes for excitation and emission of 16 nm. Quantum yields were determined by means of the gradient method, using the dye DCM excited at 450 nm ($\Phi_{em}=0.6\pm0.04$) as a standard.[19]

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[1] a) M. J. Heller, *Annu. Rev. Biomed. Eng.* **2002**, *4*, 129–153; b) A. Sassolas, B. D. Leca-Bouvier, L. J. Blum, *Chem. Rev.* **2008**, *108*, 109–139.

[2] a) E. Paleček, F. Jelen in *Electrochemistry of nucleic acids and proteins: Towards electrochemical sensors for genomics and proteomics* (Eds.: E. Paleček, 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. Paleček, F. Scheller, J. Wang), Elsevier, Amsterdam, **2005**, pp. 175–194.

[3] E. Paleček, M. Fojta, *Talanta* **2007**, *74*, 276–290.

[4] V. Balzani, A. Juris, M. Venturi, *Chem. Rev.* **1996**, *96*, 759–833.

[5] a) A. A. Gorodetsky, J. K. Barton, *Langmuir* **2006**, *22*, 7917–7922; b) P. K. Bhattacharya, H. J. Lawson, J. K. Barton, *Inorg. Chem.* **2003**, *42*, 8811–8818; c) C. Stinner, M. D. Wightman, S. O. Kelley, M. G. Hill, J. K. Barton, *Inorg. Chem.* **2001**, *40*, 5245–5250; d) J. L. Kisko, J. K. Barton, *Inorg. Chem.* **2000**, *39*, 4942–4949; e) R. E. Holmlin, J. A. Yao, J. K. Barton, *Inorg. Chem.* **1999**, *38*, 174–189; f) S. J. Franklin, C. R. Treadway, J. K. Barton, *Inorg. Chem.* **1998**, *37*, 5198–5210.

[6] a) B. Elias, F. Shao, J. K. Barton, *J. Am. Chem. Soc.* **2007**, *129*, 1152–1153; b) F. Shao, J. K. Barton, *J. Am. Chem. Soc.* **2007**, *129*, 14733; c) C. J. Murphy, M. R. Arkin, Y. Jenkins, N. D. Ghatlia, S. H. Bossmann, N. J. Turro, J. K. Barton, *Science* **1993**, *262*, 1025–1029; d) T. J. Meade, J. F. Kayyem, *Angew. Chem.* **1995**, *107*, 358–360; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 352–354; e) P. J. Dandliker, R. E. Holmlin, J. K. Barton, *Science* **1997**, *275*, 1465–1468; f) E. Meggers, D. Kusch, B. Giese, *Helv. Chim. Acta* **1997**, *80*, 640–652.

[7] A. A. Gorodetsky, O. Green, E. Yavin, J. K. Barton, *Bioconjugate Chem.* **2007**, *18*, 1434–1441.
[8] a) D. J. Hurley, Y. Tor, *J. Am. Chem. Soc.* **2002**, *124*, 3749–3762; b) D. J. Hurley, Y. Tor, *J. Am. Chem. Soc.* **2002**, *124*, 13231–13241; c) D. J. Hurley, Y. Tor, *J. Am. Chem. Soc.* **1998**, *120*, 2194–2195; d) D. J. Hurley, S. E. Seaman, J. C. Mazura, Y. Tor, *Org. Lett.* **2002**, *4*, 2305–2308.
[9] H. Weizman, Y. Tor, *J. Am. Chem. Soc.* **2002**, *124*, 1568–1569.
[10] 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.
[11] M. Vrábel, R. Pohl, B. Klepetářová, I. Votruba, M. Hocek, *Org. Biomol. Chem.* **2007**, *5*, 2849–2857.
[12] Recent examples: a) 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; b) L. H. Thoresen, G.-S. Jiao, W. C. Haaland, M. L. Metzker, K. Burgess, *Chem. Eur. J.* **2003**, *9*, 4603–4610; c) S. Jäger, G. Rasched, H. Kornreich-Leshem, M. Engeser, O. Thum, M. Famulok, *J. Am. Chem. Soc.* **2005**, *127*, 15071–15082; d) 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; e) G. A. Burley, J. Gierlich, M. R. Mofid, H. Nir, S. Tal, Y. Eichen, T. Carell, *J. Am. Chem. Soc.* **2006**, *128*, 1398–1399; f) A. Shoji, T. Hasegawa, M. Kuwahara, H. Ozaki, H. Sawai, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 776–779; g) P. M. E. Gramlich, S. Warncke, J. Gierlich, T. Carell, *Angew. Chem.* **2008**, *120*, 3491–3493; *Angew. Chem. Int. Ed.* **2008**, *47*, 3442–3444; h) M. Fischler, A. Sologubenko, J. Mayer, G. Clever, G. Burley, J. Gierlich, T. Carell, U. Simon, *Chem. Commun.* **2008**, 169–171. Recent review: i) S. H. Weisbrod, A. Marx, *Chem. Commun.* **2008**, 5675–5685.
[13] P. Čapek, R. Pohl, M. Hocek, *Org. Biomol. Chem.* **2006**, *4*, 2278–2284.
[14] Recent review: M. Hocek, M. Fojta, *Org. Biomol. Chem.* **2008**, *6*, 2233–2241.
[15] P. Čapek, H. Cahová, R. Pohl, M. Hocek, C. Gloeckner, A. Marx, *Chem. Eur. J.* **2007**, *13*, 6196–6203.
[16] P. Brázdilová, M. Vrábel, R. Pohl, H. Pivoňková, L. Havran, M. Hocek, M. Fojta, *Chem. Eur. J.* **2007**, *13*, 9527–9533.
[17] H. Cahová, L. Havran, P. Brázdilová, H. Pivoňková, R. Pohl, M. Fojta, M. Hocek, *Angew. Chem.* **2008**, *120*, 2089–2092; *Angew. Chem. Int. Ed.* **2008**, *47*, 2059–2062.
[18] H. Cahová, R. Pohl, L. Bednárová, K. Nováková, J. Cvačka, M. Hocek, *Org. Biomol. Chem.* **2008**, *6*, 3657–3660.
[19] M. Vrábel, R. Pohl, I. Votruba, M. Sajadi, S. A. Kovalenko, N. P. Ernsting, M. Hocek, *Org. Biomol. Chem.* **2008**, *6*, 2852–2860.
[20] T. Kovacs, L. Ötvös, *Tetrahedron Lett.* **1988**, *29*, 4525–4528.
[21] a) M. G. McDougall, L. P. Hosta, S. Kumar, C. W. Fuller, *Nucleosides Nucleotides* **1999**, *18*, 1009–1011; b) M. G. McDougall, L. Sun, I. Livshin, L. P. Hosta, B. F. McArdle, S.-B. Samols, C. W. Fuller, S. Kumar, *Nucleosides Nucleotides Nucleic Acids* **2001**, *20*, 501–506.
[22] M. Fojta, P. Kostečka, M. Trefulka, L. Havran, E. Paleček, *Anal. Chem.* **2007**, *79*, 1022–1029.
[23] I. V. Yang, P. A. Ropp, H. H. Thorp, *Anal. Chem.* **2002**, *74*, 347–354.
[24] V. Mirceski, S. Komorski-Lovric, M. Lovric, *Square-Wave Voltammetry. Theory and Application*, Springer, Berlin, **2008**.
[25] J. Wang, G. Liu, A. Merkoci, *J. Am. Chem. Soc.* **2003**, *125*, 3214–3215.
[26] E. M. Kober, J. V. Caspar, B. P. Sullivan, T. J. Meyer, *Inorg. Chem.* **1988**, *27*, 4587–4598.
[27] a) M. Fojta, L. Havran, R. Kizek, S. Billova, *Talanta* **2002**, *56*, 867–874; b) M. Fojta, L. Havran, S. Billova, P. Kostečka, M. Masarik, R. Kizek, *Electroanalysis* **2003**, *15*, 431–440.

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