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Redox-Active Metal-Containing Nucleotides: Synthesis, Tunability, and Enzymatic Incorporation into DNA

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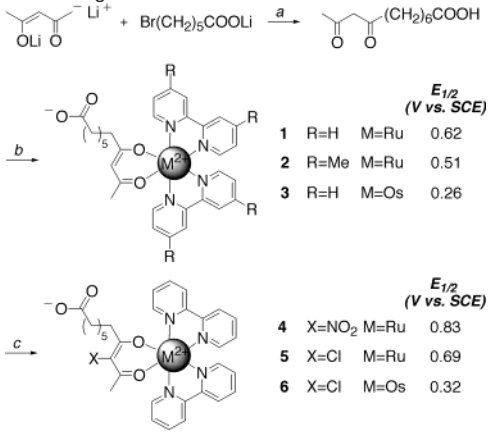
Recent developments in "lab-on-a-chip" approaches and DNA diagnostic tools have triggered a renewed interest in the incorporation of electrochemically active modifications into oligonucleotides.[1,2] This has been motivated by the emerging need to develop nonoptical detection techniques that are amenable to miniaturization.[3,4] Early approaches to redox-active DNA tags utilized organic probes (e.g., anthraquinones),[5] while more recent systems have relied on ferrocene-containing nucleosides.[6,7] Two important advances remain to be addressed: (1) The development of multiple tags with diverse and "tunable" redox potentials that maintain similar structural and chemical features, and (2) the development of redox-active nucleoside triphosphate that can be enzymatically incorporated at any position by DNA polymerase.[8] Here we disclose the design, synthesis, and characterization of novel redox-active tags with tunable electrochemical potentials, as well as the synthesis and successful enzymatic incorporation of an electrochemically active nucleoside triphosphate into DNA oligonucleotides.

Several criteria have been taken into consideration when designing electrochemically active nucleotides and DNA-labeling agents. The $E_{1/2}$ value of the electroactive moiety should fall outside the oxidation or reduction potentials of the natural heterocyclic bases.[9] Taking into account the oxidation and reduction potentials of water and the diminished barrier for oxidation of G residues when found within G-tracts, useful electrochemically active DNA labels should possess redox potentials between +0.8 and −0.7 V versus SCE.[10] Within this window, a range of redox potentials should be accessible to ensure well-resolved signals for "multicolor" detection. The electroactive moieties should also be chemically stable and compatible with polymerase reaction conditions and electrophoretic separations.

Kinetically inert polypyridine Ru^{2+} and Os^{2+} complexes are attractive candidates due to their chemical stability and favorable redox properties: the $M^{n/n+1}$ couples are metal-centered and reversible, and their $E_{1/2}$ can be tuned by the selection of the coordinated ligands.[11] $[Ru(bpy)_3]^{2+}$ and $[Ru(phen)_3]^{2+}$ exhibit oxidation potentials outside the proposed working range ($E_{1/2} \approx 1.25$ V vs SCE).[11] If one of the polypyridine ligands, however, is replaced by a negatively charged ligand (e.g., an acetylacetonate or hydroxamate), the resulting complexes $[(bpy)_2Ru(L)]^+$ exhibit appreciably lower oxidation potentials, well within the desired range.[11,12]

The synthesis of the tagged nucleotides is modular and encompasses the following building blocks: (a) substituted 2,4-pentanediones or hydroxamic acids bearing a functionalized linker, (b) bis-substituted Ru^{2+} or Os^{2+} precursors $(R_2bpy)_2ML_2$, and (c) modified nucleotides. Alkylating the dianion derived from acetylacetone with lithium 5-bromopropanoate affords a functional linker (Scheme 1).[13] Treating the resulting ligand with bis-substituted bipyridine Ru^{2+} or Os^{2+} precursors gives the neutral complexes **1**−**3**.[13] Complexation with metal ions activates the acetylacetonate and facilitates

Scheme 1. Synthesis and Electrochemical Data of Acetyacetonate Complexes Bearing a Functionalized Linker for Nucleotide Tagging[a]



[a] Reagents and Conditions: (a) THF, −78 °C to rt, 29%; (b) $(R_2bpy)_2MCl_2$, $EtOH/H_2O$, $CaCO_3$, reflux, 80−95%; (c) For **4**: $Cu(NO_3)_2/Ac_2O$, 71%; for **5** and **6**: *N*-chlorosuccinimide, 58−60%.[13]

electrophilic substitution reactions (e.g., halogenation and nitration) at the central position to give complexes **4**−**6** (Scheme 1).[14]

Cyclic voltammetry of the complexes shows a reversible wave for the metal centered oxidation with $\Delta E = 68 \pm 2$ mV (CH_3CN, 0.1 M n-$Bu_4N^+PF_6^-$), close to the anticipated 1*e* Nerstian process.[13] As expected, oxidation of the parent ruthenium complex **1** occurs at considerably lower potentials when compared to $[Ru(bpy)_3]^{2+}$, due to the lower charge and stabilization of the higher oxidation state by the O,O-donor ligands.[15] The tunabilty of redox potentials is demonstrated by substituting the bipyridine co-ligands, selecting other central ions and modifying the acetonate moiety. Replacing the bpy co-ligands with 4,4′-dimethyl-2,2′-bipyridine results in a shift of 0.11 V to lower potentials. A more pronounce negative shift of 0.36 V is observed for the analogous osmium complex **3** (Scheme 1). Modifying the complexes with electron-withdrawing substituents (e.g., NO_2 in **4** and Cl in **5** and **6**) results in a shift to more positive potentials.

Functionalized hydroxamic acids are synthesized by alkylating benzyl-4-hydroxybenzoate with a suitable spacer followed by activation as the pentachlorophenyl ester (Scheme 2).[13] Condensation with *N*-methyl hydroxylamine provides the *N*-substituted hydroxamic acid. Complexation with metal bipyridine carbonate precursors gives complexes **7**−**9**.[16] The hydroxamate complexes exhibit a reversible redox waves and their $E_{1/2}$ values are negatively shifted compared to those for the analogous acetylacetonates. The effect of changing the co-ligands and metal ions is similar to that observed for the acetonate complexes shown in Scheme 1. Together with the acetylacetonate derivatives, complexes **1**−**9** span a potential window of approximately 0.9 V.

A redox-active dTTP analogue **10** is synthesized by coupling 5-(3″-aminopropynyl)-2′-deoxyuridine-5′-triphosphate to the suc-

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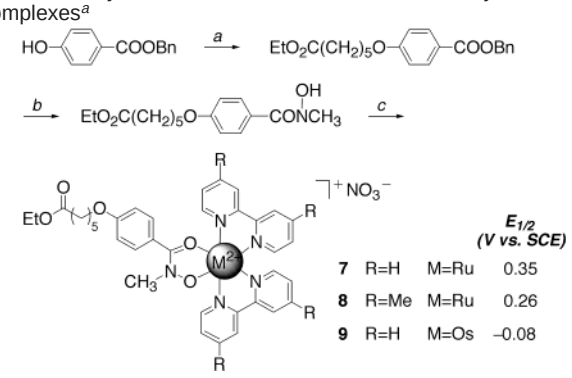
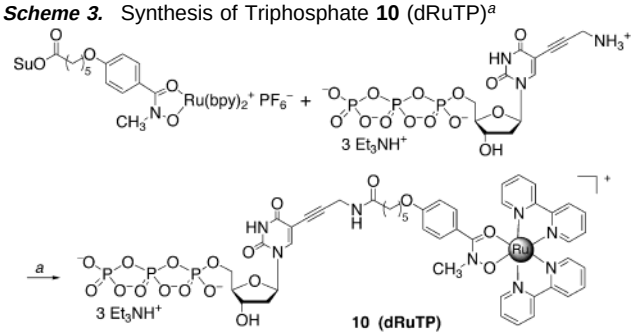
Scheme 2. Synthesis and Electrochemical Data of Hydroxamate Complexes[a]



[a] Reagents and Conditions: (a) $Br(CH_2)_5CO_2Et$, NaH, THF, −78 °C to rt, 88%; (b) (i) H_2/Pd, 92%, (ii) C_6Cl_5OH, DCC, 85%, (iii) $NH(OH)CH_3$, DMAP, 59%; (c) $(R_2bpy)_2MCO_3$, 30−52%.

Scheme 3. Synthesis of Triphosphate **10** (dRuTP)[a]



[a] Reagents and conditions: DMF/dioxane/H_2O, (iPr_2)NEt, quantitative.

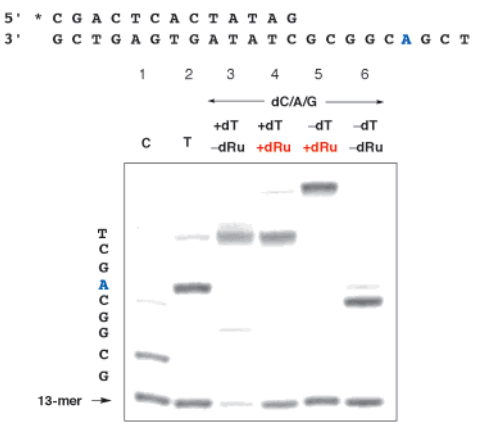


Figure 1. Enzymatic incorporation of a Ru^{2+} modified nucleotide dRuTP. (Top): Template and ^{32}P-labeled primer oligonucleotides. (Bottom): Primer extension experiments using the Klenow fragment of DNA polymerase resolved on a 20% PAGE. (Lanes 1 and 2): C and T sequencing lanes, respectively. (Lane 3): Full-length product is generated in the presence of all triphosphates. (Lane 4): No deleterious effect is observed in the presence of all triphosphate and dRuTP. (Lane 5): Full-length product containing a single Ru-containing base is observed. (Lane 6): Truncated product is generated in the absence of dTTP and dRuTP.

cinimide active ester of the metal complex (Scheme 3).[13] The enzymatic incorporation of the modified base **10** was tested using a 5′-^{32}P-labeled 13-mer primer and a 22-mer template (Figure 1).[17] Primer extension in the presence of all four dNTPs gives the expected full-length product (lane 3). Addition of dRuTP to the four dNTPs does not interfere with the enzymatic elongation process (lane 4). If dTTP is eliminated, premature termination occurs right after the CGGC site, yielding an 18-mer product (lane 6). When dTTP is replaced by dRuTP, only one band appears, and premature termination is not observed (lane 5).[18] The full-length 22-mer metal-containing DNA displays a slower electrophoretic mobility when compared to the corresponding native fragments due to the increased mass and additional single positive charge at the metal center. Full-length extension is also observed with a longer template containing multiple dA residues.[19] Taken together, these experiments demonstrate that the modified nucleotide is very well tolerated by the polymerase at the triphosphate level as well as part of the growing chain.[20]

These results demonstrate that octahedral Ru^{2+} and Os^{2+} complexes are viable, diverse, and tunable redox-active tags for DNA modification. The ability of DNA polymerase to efficiently incorporate the metal-containing nucleotide triphosphate suggests potential use in DNA diagnostics.

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Supporting Information Available: Additional synthetic, analytical, and electrochemical data as well as details for enzymatic synthesis of oligonucleotides (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Converting the terminal carboxylate to benzylamide does not affect the $E_{1/2}$ value of the complex.[13]
(16) An analogous complex to **7**, in which the hydroxamic acid is not substituted [i.e., R-CON(OH)**H**], has been found to be unstable.[13]
(17) The template contains a single adenosine residue to unequivocally determine the incorporation of the modified nucleotide and the generation of a full-length product.
(18) A similar faint band also appears when dRuTP is mixed with dTTP in a 1:1 ratio (compare lanes 4 and 5), suggesting that the modified nucleotide can compete to an extent with the native nucleotide for incorporation opposite dA.
(19) Template: GCT-GAG-TGA-TAT-CGC-**A**GC-**A**TC-**A**GT-**A**CC.
(20) The ability of a polymerase to accept a modified dNTP as a substrate and extend the growing chain beyond the modification site is significantly more demanding than end labeling or chain termination.

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