Solid-Phase Synthesis and Photophysical Properties of DNA Labeled at the Nucleobase with $Ru(bpy)_2(4\text{-m-}4'\text{-pa-bpy})^{2+}$

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A facile procedure for incorporating a $Ru(diimine)_3^{2+}$ complex at the nucleobase in an oligonucleotide is reported that combines the advantages of Pd(0) cross-coupling and solid-phase DNA chemistries. These ruthenium-modified oligonucleotides form stable duplexes, and the favorable photophysical properties associated with the $Ru(diimine)_3^{2+}$ complex are retained after site-specific covalent attachment.

Introduction

Oligonucleotides labeled with spectroscopic or functional probes are of widespread interest for clinical applications and basic research.[1−14] Transition metal complexes are finding ever increasing roles in these areas.[15−20] However, the ability to site-specifically modify an oligonucleotide with such chemical functionalities is limited by the current synthetic procedures. Previous methods toward metal-derivatized oligonucleotides focused on either modifying the nucleic acid single strand after solid-phase synthesis or synthesizing the labeled phosphoramidite for subsequent incorporation in the nucleic acid strand.

This first approach, attaching metals to oligonucleotides after solid-phase synthesis, is used by a number of research groups.[17,18,21−29] This postmodification strategy requires selective solution- or solid-phase coupling of a metal complex or metal with the oligonucleotide. For example, in 1989, Netzel reported the synthesis of oligonucleotides labeled with $Ru(bpy)_3^{2+}$ at the nucleobase.[23] A 5-aminouridine nucleoside was synthesized and incorporated in an oligonucleotide. The purified and deprotected oligonucleotide was then reacted with succinimidyl-4-carboxy-4′-methyl-2,2′-bipyridine to form the oligonucleotide−bpy complex and subsequently reacted with $Ru(bpy)_2Cl_2$ to form the ruthenium labeled oligonucleotide. The other common labeling site is the 5′-terminal phosphate of the oligonucleotide. The groups of Bannwarth, Barton, and Sessler have used this procedure to synthesize oligonucleotides labeled with metal complexes.[17,24,25,27] Once again, the amino-modified oligonucleotide was reacted with a succinimidyl ester derivative of the metal complex to form the metallo-oligonucleotide. To introduce greater binding specificity to a metal, the groups of Dervan, Sigman, Bergstrom, and Bashkin have attached multidentate ligands (e.g., EDTA, phenanthroline, and terpyridine) to the nucleobase and incorporated these modified nucleosides in oligonucleotides for subsequent metal binding in solution.[18,21,22,29,30] These postmodification couplings of the metal complex or metal with a modified oligonucleotide, however, are often hampered by low coupling yield and multiple side reactions.

An alternative strategy that offers several advantages over the previous approach is to exploit standard automated DNA solid-phase synthetic methods for the site-specific incorporation of a transition metal complex in an oligonucleotide. A number

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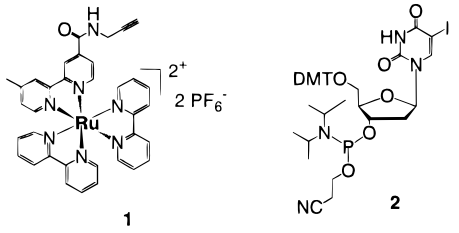
of groups, including ours, are currently exploring this synthetic route.[21,31−38] This approach was demonstrated by Bannwarth in 1989, who attached a Ru(diimine)$_3$ phosphoramidite to the terminal 5′-hydroxyl of an oligonucleotide.[33] The synthesis of metallooligonucleotides using ruthenium derivatized uridine phosphoramidites has been reported by two groups.[35,37] Tor reported the synthesis of a Ru(phen)$_3$-modified uridine in good yield through two successive Pd(0) cross-coupling reactions, starting from 3-bromophenanthroline. The coupling of this ruthenium phosphoramidite to the oligonucleotide was then performed separately and manually from the normal operation on the DNA synthesizer. We synthesized a 5-[Ru(bpy)$_2$(4-m-4′-pa-bpy)]$^{2+}$-2′-deoxyuridine phosphoramidite and incorporated this derivatized uridine in an oligonucleotide using a standard reaction coupling protocol on an automated DNA synthesizer.[37] We selected this particular 5-[Ru(bpy)$_2$(4-m-4′-pa-bpy)]$^{2+}$-2′-deoxyuridine nucleoside since the excited state was known to be long-lived and localized on the bipyridine attached to the uridine.[39] This phosphoramidite approach is suited for synthesizing metallo-labeled oligonucleotides; however, it often requires the synthesis of large molecular weight nucleoside derivatives containing complex functional groups that are time-consuming to prepare and difficult to purify and handle.

Thus, there is a need for alternative methods of labeling oligonucleotides at site-specific locations with transition metal complexes that can serve as synthetic nucleases, cross-linking agents, or spectroscopic probes. Herein, we report the synthesis of a series of Ru(diimine)$_3^{2+}$-labeled oligonucleotides using an *on-column derivatization* method that combines the advantages of Pd(0) cross-coupling and solid-phase DNA chemistries as shown in Scheme 1.[40] We also describe the photophysical properties of these single- and double-stranded ruthenium-labeled oligonucleotides.

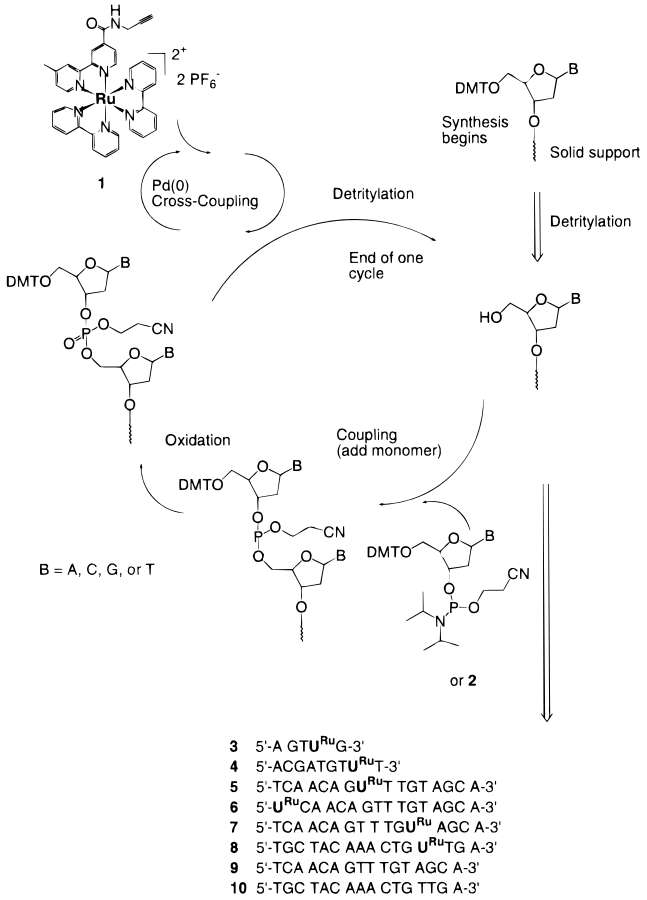
Results and Discussion

This *on-column derivatization* method requires an alkynyl-functionalized ruthenium complex and a 5-iodo-substituted nucleobase in the oligonucleotide for Sonogashira Pd(0) cross-coupling[41] during automated DNA synthesis.[42−44] The (bpy)$_2$-(4-m-4′-pa-bpy)ruthenium(II) bis(hexafluorophosphate), **1**, and 5′-dimethoxytrityl-3′-(β-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite)-2′-deoxy-5-iodouridine, **2**, are shown in Chart 1. The synthetic procedure to incorporate **2** and the phosphoramidites of A, C, G, and T in a nucleic acid single strand using standard automated DNA synthesis is shown in Scheme 1. Next, the

Chart 1



Scheme 1



5-iodouridine is cross-coupled with the alkynyl-functionalized trisbipyridine ruthenium complex, **1**, using Pd(PPh$_3$)$_4$ and CuI. After the oligonucleotide is labeled on-column, DNA synthesis is resumed and additional A, C, G, or T bases are added to complete the sequence. Finally, the protected oligonucleotide is cleaved from the column and incubated at 55 °C in NH_3 for 16 h to completely deprotect the oligonucleotide. The ruthenium-labeled oligonucleotide is then purified by HPLC. The retention times of the labeled oligonucleotides are well separated from the unlabeled oligonucleotide products (>3 min in retention time).

A series of short and long site-specifically Ru(bpy)$_2$(4-m-4′-pa-bpy)$^{2+}$-labeled oligonucleotides are synthesized using this method (Scheme 1). This procedure is also amenable to the synthesis of oligonucleotides labeled with Ru(bpy)$_2$(4-m-4′-pa-bpy)$^{2+}$ at various positions in the sequence. As shown in Figure 1, thermal denaturation experiments on unlabeled (**9·10)** and Ru(bpy)$_2$(4-m-4′-pa-bpy)$^{2+}$-labeled oligonucleotide duplexes (**5·10)** show no substantial change in thermal stability when the ruthenium complex is introduced in the middle of the oligonucleotide sequence: the melting temperature (T_m) of the unmodified duplex **9·10** is 49 °C compared to 48 °C for the

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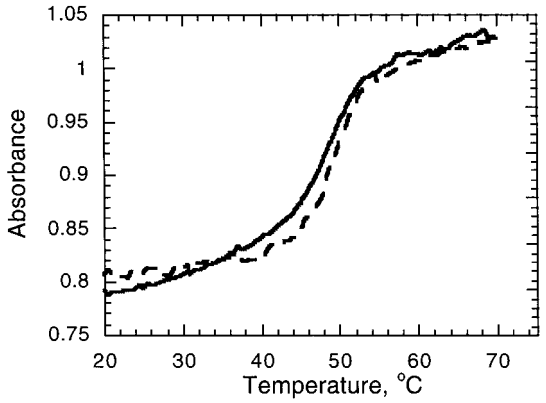

Figure 1. Melting curve profiles for $Ru(diimine)_3^{2+}$ labeled (**5·10**, dashed line) and unlabeled (**9·10**, solid line) oligonucleotides.

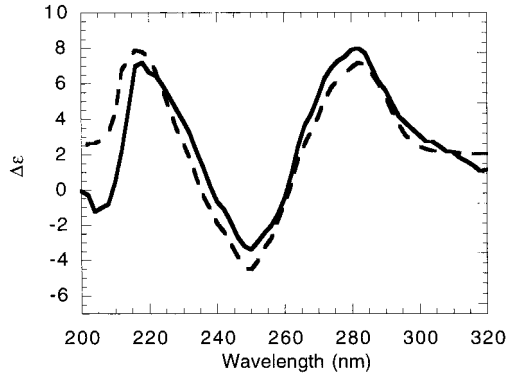

Figure 2. CD spectra of unlabeled (**9·10**, dashed line) and $Ru(diimine)_3^{2+}$-labeled (**5·10**, solid line) oligonucleotide duplexes.

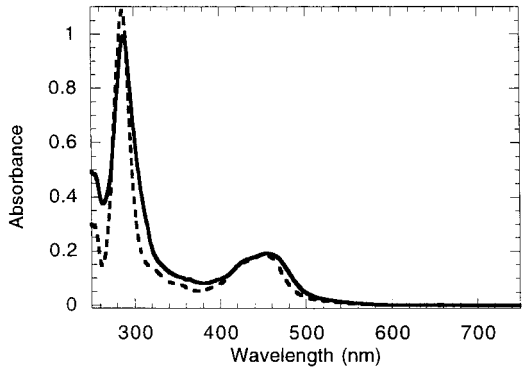

Figure 3. Electronic absorption of $Ru(bpy)_3^{2+}$ (dashed line) and **1** (solid line) in CH_3CN.

ruthenium-labeled duplex **5·10**. When the metal complex is attached to the terminal nucleotide of the sequence, the T_m is unchanged (**6·10**, $T_m = 49$ °C). Circular dichroism (CD) spectra further support formation of a well-defined B-form DNA duplex.[45] CD spectra of the unlabeled (**9·10**) and $Ru(diimine)_3^{2+}$-labeled (**5·10**) oligonucleotides are similar, and the characteristic spectral features for B-DNA are present (Figure 2).

The alkynyl-derivatized trisbipyridine ruthenium complex, **1**, exhibits the characteristic metal-to-ligand charge-transfer band ($^1MLCT\text{-}^1A_1$), centered at 450 nm in the absorption spectrum, analogous to that of $Ru(bpy)_3^{2+}$ (Figure 3). The bipyridine $\pi-\pi^*$ transitions are observed at 280 nm. Excitation of the

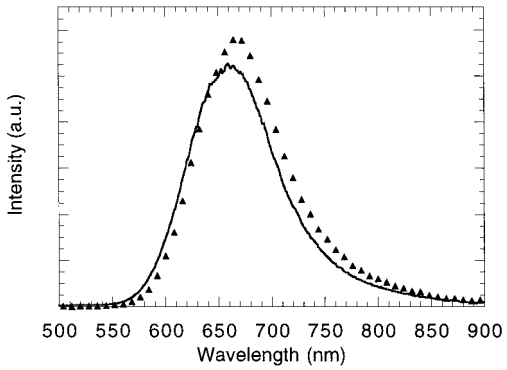

Figure 4. Emission spectra of **1** (solid triangles) and duplex **5·10** (solid line), in phosphate buffer (5 mM sodium phosphate, NaCl 50 mM, pH 7.0, 10^{-6} M chromophore and DNA concentrations). Corrected emission spectra recorded at 298 K (excitation at 450 nm).

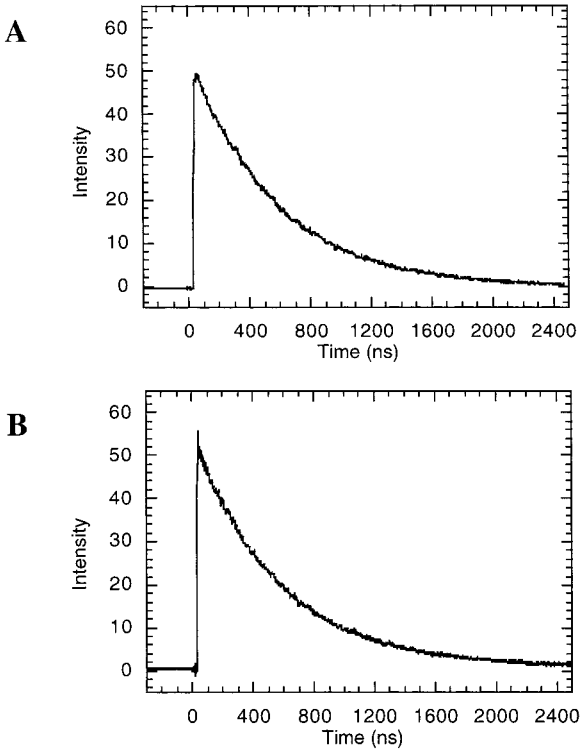

Figure 5. Emission decay traces monitored at 650 nm for $Ru(diimine)_3^{2+}$-labeled single-strand **5** (A) and modified duplex **5·10** (B), after 460 nm pulse excitation (10^{-6} M DNA concentrations).

MLCT band produces an emission maximum centered at 666 nm in aqueous solution, and slightly red-shifted relative to that of $Ru(bpy)_3^{2+}$ (625 nm). Transient absorption spectra obtained by exciting a degassed solution of **1** in CH_3CN with a 460 nm laser pulse reveals the characteristic absorption band for the ruthenium trisbipyridine radical anion at 380 nm.

Once **1** is incorporated in the oligonucleotide, the emission maximum shifts ~6 nm to higher energy compared to that of the free complex **1** (Figure 4). The emission maximum for the modified single-strand and duplex oligonucleotides are both centered at 660 nm. The emission lifetime of this ruthenium-labeled oligonucleotide **5** is 544 ns, and that of the modified oligonucleotide duplex **5·10** is 594 ns at 25 °C in phosphate buffer (parts A and B, respectively, of Figure 5). The characteristic absorption band for the ruthenium trisbipyridine radical anion at 380 nm is also observed in the transient absorption spectra of the ruthenium-labeled oligonucleotide duplex. The $Ru(bpy)_2(4\text{-m-}4'\text{-pa-bpy})^{2+}$ excited-state lifetime is only slightly

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longer for the ruthenium-labeled duplex compared to that of the single strand, indicating that hybridization does not substantially alter the electronic structure or local environment of the $Ru(diimine)_3^{2+}$. Importantly, quenching of the $Ru(bpy)_2(4\text{-m-}4'\text{-pa-bpy})^{2+}$ excited state is not observed, consistent with the known redox potentials of A, C, G, and T.[46]

Conclusion

In summary, a facile solid-phase procedure for the site-specific labeling of an oligonucleotide at the nucleobase with a substitutionally inert, coordinatively saturated metal complex during automated DNA synthesis is reported. The advantages of this *on-column derivatization* method over the existing postmodification and phosphoramidite strategies to metallo-oligonucleotides include (1) fewer overall synthetic steps, (2) efficient Pd(0) cross-coupling reactions, (3) practical solid-phase reaction conditions, (4) ease of oligonucleotide purification and isolation, and (5) precise control over the location of the $Ru(diimine)^{3+}$ complex in the oligonucleotide sequence. These ruthenium-labeled oligonucleotides form stable B-form duplexes at room temperature, and the photophysical properties of the ruthenium complex are retained after site-specific covalent attachment.

Experimental Section

Abbreviations: bpy = 2,2′-bipyridine; 4-m-4′-pa-bpy = 4-methyl-2,2′-bipyridine-4′-carbonylpropargylamine; HOBt = 1-hydroxybenzotriazole; DIPEA = *N*,*N*-diisopropylethylamine; DCC = dicyclohexylcarbodiimide; DMT = dimethoxytrityl.

All solvents were dried and distilled prior to use. Absorption spectra were measured on a Hewlett-Packard 8452 diode array spectrometer. Emission spectra were recorded on a Perkin-Elmer LS50B or a Spex Fluorolog-2 emission spectrometer. CD spectra were recorded on a Aviv 202 CD spectropolarimeter. Reversed-phase HPLC was performed on a Rainin HPLC with a C18 column monitoring at 254 and/or 450 nm.

Syntheses. Bis(2,2′-bipyridine)(4-methyl-2,2′-bipyridine-4′-carbonylpropargylamine)ruthenium(II) bis(hexafluorophosphate), **1**, was synthesized as previously described.[39] 5′-(4,4′-Dimethoxytrityl)-3′-(β-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite)-2′-deoxy-5-iodouridine, **2**, was synthesized following the published procedure of Caruthers.[47] This iodouridine phosphoramidite is also commercially available from Glen Research.

Oligonucleotide Syntheses. The oligonucleotide syntheses were performed on a commercial ABI 395 DNA/RNA synthesizer from the 3′ to 5′ end using standard automated DNA synthesis protocols as shown in Scheme 1 (0.2 and 1.0 μmol scale). A 0.1 M solution of 5′-DMT-3′-(β-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite)-2′-deoxy-5-iodouridine in dry acetonitrile was prepared and installed on the DNA synthesizer in a standard reagent bottle. All solid-phase syntheses were performed in such a manner that the synthesis cycle was paused after incorporation of the 5′-DMT-3′-(β-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite)-2′-deoxy-5-iodouridine, without deprotecting the 5′-hydroxyl or cleaving the oligonucleotide from the resin. The column was subsequently removed from the synthesizer and sparged with N_2 to maintain the anhydrous condition. Next, the alkyne-derivatized functional group (6 μmol), $Pd(Ph_3P)_4$ (6 μmol), CuI (3 μmol), and 150 μL of dry solvent DMF/Et_3N (3.5:1.5) were added, and the column was placed on a shaker for 3 h. Then the column was washed with DMF/Et_3N (9:1; 10 mL) and acetonitrile (40 mL), dried with N_2 for 30 min, and reinstalled on the synthesizer. Solid-phase synthesis was resumed, and additional DNA bases were added. The purified ruthenium-modified oligonucleotide exhibited one peak in an HPLC trace, with retention times greater than those of the corresponding unmodified oligonucleotide. MALDI or electrospray mass spectrometry of the metallooligonucleotide confirmed formation.

Collection and analysis of the DMT fractions during automated synthesis showed efficient phosphoramidite coupling throughout the procedure, with both the standard pyrimidine and purine nucleosides as well as with 5-iodouridine (>95%). Analysis of the HPLC traces of the crude oligonucleotide products showed efficient Pd(0) cross-coupling reactions for **1** (final yields ranged from 75% to 92%).

HPLC Purification of the Oligonucleotides. HPLC purification of the modified oligonucleotides was accomplished on a Rainin HPLC instrument. Reverse-phase chromatography was performed on a C18 column (25 cm × 4.6 mm) with acetonitrile (ACN) and aqueous 0.1 M triethylamine acetate (TEAA) as eluting solvents. A flow rate of 1 mL/min was used, and the concentration of ACN was increased from 5% to 50% over 50 min. The retention times of the modified oligonucleotides were well separated from the unmodified oligonucleotide products (>3 min in retention time).

Melting Curves. The stability of the duplex formed between the two complementary oligonucleotides was determined by analyzing the melting curve profile as a function of temperature. Briefly stated, 2 mM stock solutions of the separate oligonucleotides were prepared and diluted to a working solution of 0.5 absorbance unit (5 mM phosphate; 50 mM sodium chloride; pH 7). Next the two solutions were combined, and the solution containing the complementary oligomers was heated to 90 °C for 5 min. The solution was then allowed to cool to room temperature over 3 h. After cooling, the thermal denaturation experiment was performed using the following parameters on a HP8452 UV−vis spectrophotometer: (a) monitoring wavelength 260 nm, (b) temperature range 20−70 °C, (c) temperature step 0.5 °C, (d) equilibrium time 1 min.

Transient Absorbance. A Surelite II-10 (continuum) Nd:YAG-OPO system was used as the excitation source. The excitation beam from the laser irradiated the sample perpendicular to an optical axis of an Applied Photophysics laser kinetic spectrometer with a 250 W pulsed Xe lamp, f3.4 monochromator, and Hammamatsu PMT. The output from the PMT was coupled to a LeCroy 7200A oscilloscope and analyzed. Electronic synchronization and control of the experiment were achieved by electronics of local design. The excitation wavelength was 460 nm, and the power at the sample was 40 μJ/pulse as measured by a Molectron J3-09 power meter.

Emission Spectra. Corrected emission spectra were recorded on a Spex Fluorolog-2 emission spectrometer equipped with a 450 W Xe lamp and cooled Hammamatsu R928 photomultiplier. The recorded emission spectra were corrected for spectrometer response. The calibration curve was obtained using a NIST calibrated standard lamp (Optronics Laboratories, Inc. Model 220M), controlled with a precision current source at 6.5 W (Optronics Laboratories, Inc. Model 65) by following the procedure recommended by the manufacturer. The spectra were obtained in buffer at room temperature in a 1 cm quartz cell using right angle observation of emitted light.

Lifetimes. A Laser Photonics LN1000 Nitrogen Laser-LN102 dye laser (coumarin 460 dye, Exciton) was used as the irradiation source. The emission was monitored at a right angle using a Macpherson 272 monochromator and Hammamatsu R666-10 PMT. The signal was processed by a LeCroy 7200A transient digitizer interfaced with an IBM-PC. The excitation wavelength was 460 nm, and the monitoring wavelength was 620 nm. The power at the sample was 120 μJ/pulse as measured by a Molectron J3-09 power meter. The measured instrument lifetime response was 10 ns (fwhm). The acquired emission decay curves were analyzed by locally written software based on the Marquardt algorithm.

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