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Site-Specifically Labeled Metallo-Oligodeoxynucleotides

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Site-Specifically Labeled Metallo-Oligodeoxynucleotides

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Growing interest in site-specifically labeled metallo-oligodeoxynucleotides for diagnostic, therapeutic and mechanistic applications provides the impetus for the development of new synthetic methods that are user-friendly and amenable to large-scale production. This review describes advances in the field since the early 1980's and highlights recent solution-phase and automated solid-phase coupling procedures.

Keywords: *metallo-oligodeoxynucleotides; oligodeoxynucleotides; DNA; solid-phase synthesis; metal complexes*

Abbreviations: bpy, 2,2′-bipyridine, bpy′, 4-(carboxybutyl)-4′-methyl-2,2′-bipyridine, CD, circular dichroism, CDI, carbonyldiimidazole, CPG, controlled pore glass, DCC, dicyclohexylcarbodiimide, dip, 4,7-diphenylphenanthroline, DIPEA, diisopropylethylamine, DMF, dimethylformamide, DMT, dimethoxytrityl, DMT-dabp, dimethoxytrityldiamidobipyridine propanolamine, DNA, deoxyribonucleic acid, dpphen, 4,7-diphenylphenanthroline, dppz, dipyridophenazine, EDTA, ethylenediaminetetraacetic acid, EI, electrospray ionization, FMOC, 9-fluorenylmethoxycarbonyl, FPLC, fast protein

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liquid chromatography, hmbc, N-(2-hydroxyethyl)-4-methyl-2,2′-bipyridine-4-carboxamide, HOBt, 1-hydroxybenzotriazole, HPLC, high performance liquid chromatography, Me_2dppz, 9,10-dimethyldipyridophenazine, MS, mass spectrometry, N, nucleotide, PAGE, polyacrylamide gel electrophoresis, phen, 1,10-phenanthroline, phi, phenanthrenequinone diimine, pmbc, N-(2-propynyl)-4-methyl-2,2′-bipyridine-4-carboxamide, py, pyridine, RNA, ribonucleic acid, RP, reverse phase, tap, 1,4,5,8-tetraazaphenanthrene, TEA, triethylamine, T_m, melting temperature, TSU, N,N,N′,N′-tetramethyl(succinimido)urondium tetrafluoroborate, UV-vis, ultraviolet-visible

Deoxyribonucleic acid (DNA) plays a critical and central role in the chemistry and biology of living systems.[1] The encoding of genetic information and its transcription and translation are vital for normal physiological function. Irregularities in these processes can lead to a number of adverse health consequences. The study of the properties and biological functions of oligodeoxynucleotides encompasses a broad range of scientific expertise and skills from medicine to biochemistry to inorganic chemistry.[2–11] Since oligodeoxynucleotides do not possess chemical functional groups for detection, it is necessary to modify them with reporter molecules; such as biotin, intercalating dyes, or metal complexes. Transition metals possess a number of favorable properties for oligodeoxynucleotide study including unique chemical reactivity and photochemical activity.

Presently, there is growing interest in the design and synthesis of site-specifically labeled metallo-oligodeoxynucleotides for a variety of therapeutic, diagnostic and mechanistic applications. On the therapeutic forefront, anticancer and antisense metallo-oligodeoxynucleotides are being studied. Pt-complexed oligodeoxynucleotides are of interest as potential chemotherapeutic agents for the treatment of testicular, ovarian and other cancers.[12,13] Eu-labeled-oligodeoxynucleotides are capable of hydrolyzing their RNA complement and acting as "chemical nucleases."[14] Iron(II)·EDTA and Cu-diimine and -triimine labeled oligodeoxynucleotides are also able to cleave DNA.[15–18]

The fabrication of DNA diagnostic devices constitutes a second area sparring this research effort. Transition metals offer the possibility of spectroscopic and/or electrochemical DNA sensing. Given the current state of microelectrofabrication and oligodeoxynucleotide syntheses, devices that detect single duplexes electrochemically will likely be commercially available in the future.[19] For example, an electrochemical gene sensor is recently reported using a ferrocene-modified oligodeoxy-

nucleotide probe.[20] Today, metallo-labeled oligodeoxynucleotides are already attractive alternatives to radiolabels in the detection of viral and bacterial infections, as well as for the diagnosis of genetic diseases.[10,20–24]

Metallo-labeled oligodeoxynucleotides are also of interest for studying biophysical processes in DNA such as DNA-mediated charge transfer.[8,9,11] Mechanistic insight into radical-cation migration and electron-transfer reactions in DNA will provide a better understanding of oxidative damage *in vivo*. The covalent attachment or intercalation of metal complexes at fixed and known positions in DNA provides an opportunity to evaluate the factors affecting DNA mediated charge transfer such as oligodeoxynucleotide sequence, π-stacking, base-pairing and duplex structure, as well as donor/acceptor distance, labeling site, and dynamics. Metallo-labeled oligodeoxynucleotides also possess several advantages over the traditional organic dye labels, namely, tunable and spectroscopically distinguishable redox states as well as reversible electrochemistry.

Recent synthetic methods for the site-specific covalent attachment of metal complexes to oligodeoxynucleotides include phosphate, ribose, and nucleobase modification using both solution- and solid-phase coupling schemes, or a combination of both. In this review, we describe the current methods for labeling oligodeoxynucleotides with metal complexes and discuss the advantages and limitations of each procedure.

AUTOMATED SOLID-PHASE DNA SYNTHESIS

Solid-phase DNA synthesis was first accomplished in 1965 using strategies originally designed for peptide synthesis.[25,27] Since then, improvements in these methods[28–36] have led to the development of automated DNA synthesizers which enable researchers to easily design and synthesize DNA as large as hundreds of nucleotides in length. Automated DNA synthesis requires the use of nucleosides that are protected on the exocyclic amino groups to prevent undesired side reactions. Benzoyl groups protect the amino groups on adenosine and cytidine while the isobutyryl group is used for guanosine. The 5′-position of the ribose is protected with a dimethoxytrityl (DMT) group, which both temporarily protects the 5′-terminus and provides a means to monitor the coupling efficiency of each reaction step. The 3′-position of the ribose is modi-

fied with a phosphoramidite group, which ultimately provides the phosphodiester linkage between nucleotides.

As shown in Figure 1, the DNA synthesis cycle proceeds from the 3′- to the 5′-terminus. Nucleotide monomers are added to a nucleoside, which is bound to a solid support via the 3′-hydroxyl, through a series of reactions. The first step of the synthesis (a) is cleavage of the 5′-DMT group by trichloroacetic acid. This free 5′-hydroxyl then couples (b) to an activated monomer, formed by the addition of a nucleoside phosphoramidite and tetrazole to the solid support. Next, "capping" (c) with acetic anhydride terminates any chains that did not undergo coupling. Finally, the nucleotide linkage is oxidized from the phosphite to the phosphotriester by iodine (d), ending the first cycle. After oxidation, the 5′-DMT group is cleaved (e) and the cycle continues until the desired oligodeoxynucleotide is synthesized. Cleavage of the DMT group can be quantified spectroscopically to determine the efficiency of each coupling step. The exocyclic amino protecting groups are removed by heating the oligodeoxynucleotide in warm ammonium hydroxide. Yields for each coupling step for automated solid-phase DNA synthesis are as high as 99%.

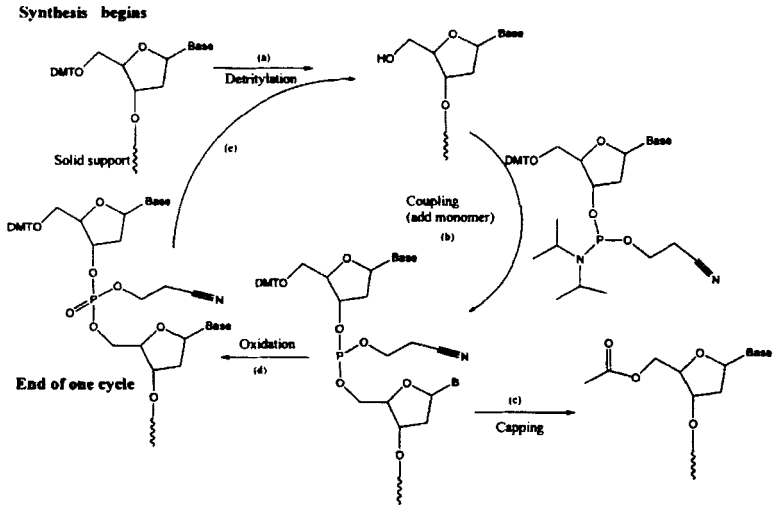


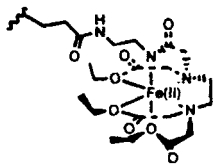
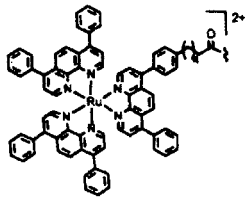
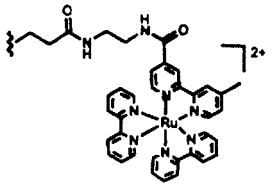
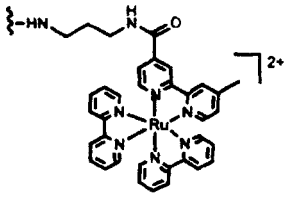
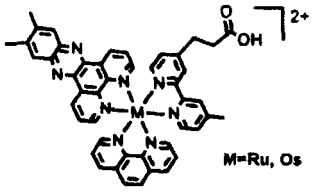
FIGURE 1 Standard automated DNA synthesis cycle.[28,35,36]

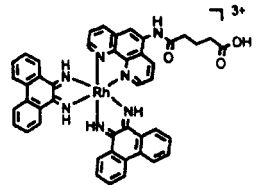
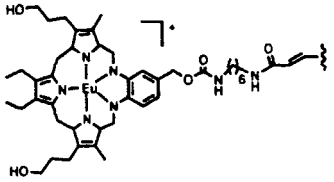
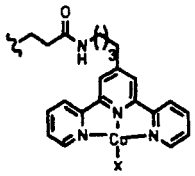
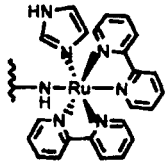
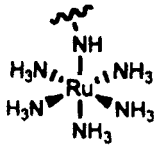
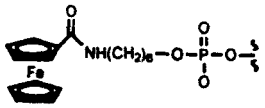
SOLUTION-PHASE LABELING

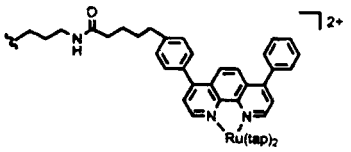
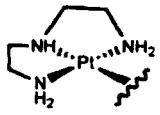
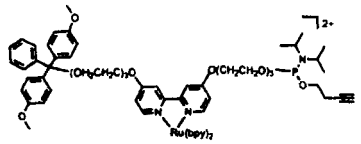
Solution-phase coupling of a metal complex to an oligodeoxynucleotide typically involves the incorporation a functional group in the oligodeoxynucleotide using an automated solid-phase DNA synthesizer, followed by reaction of the functionalized oligodeoxynucleotide with the metal or metal complex in solution (*post-modification*). Table I summarizes the research reported to date and indicates the site where the metal complex is attached. Dreyer and Dervan[15] report the synthesis of an EDTA-labeled oligodeoxynucleotide using this approach in 1985. The first step in this *post-modification* procedure requires that a linker terminated with a primary amine be attached to the C^5-position of uridine. The activated ester of EDTA (EDTA triethylester-N-hydroxysuccinimide ester) is then coupled to this amino-modified uridine to afford the EDTA-derivatized uridine. Next, the corresponding nucleoside phosphoramidite is prepared for standard automated DNA synthesis. This EDTA-modified uridine is incorporated in an oligodeoxynucleotide with a coupling efficiency of 97% (approximately 50% overall yield for a 19-mer). The single oligodeoxynucleotide strand is purified by gel electrophoresis and confirmed by Maxam-Gilbert sequencing methods. Once the EDTA-DNA duplex is formed, Fe(II) is added to form the metallo-labeled DNA. Cleavage of the DNA, which occurs at the site complementary to the DNA-EDTA probe, is initiated by the addition of a reducing agent (dithiothreitol).

Bannwarth and coworkers[37] first modify the 5′-end of an oligodeoxynucleotide with either 5′-amino-5′-deoxythymidine phosphoramidite or (2-cyanoethyl)[(diisopropyl)amino]{3-[(4-methoxytrityl)amino]propoxy}phosphine. After synthesis, the amino-labeled oligodeoxynucleotides are cleaved from the column and deprotected, then dialyzed twice against KCl. The crude oligodeoxynucleotides then couples to the N-hydroxysuccinimide ester of a ruthenium tris-diimine complex. Isolation and purification of the ruthenium-labeled product is achieved on HPLC. The ruthenium-oligodeoxynucleotides bind specifically to their complementary strand and undergo elongation by T7 DNA polymerase when hybridized.

TABLE I Metallo-oligonucleotides synthesized by solution-phase coupling

Research Group	*Ref.*	*Attachment Site*	*Metal Complex*
Dervan	15	C^5-thymidine	
Bannwarth	37	5′-phosphate	
Netzel	23	C^5-thymidine	
		C^4-cytidine	
Barton	38,40	5′-phosphate	

Research Group	*Ref.*	*Attachment Site*	*Metal Complex*
Barton	39	5′-phosphate	
Sessler	14	5′-phosphate	
Bashkin	18	C^5-thymidine	
Meade	41	C^5-thymidine	 
Ihara	21	5′-terminus	

Research Group	*Ref.*	*Attachment Site*	*Metal Complex*
Ortmans	42	C^5-thymidine	
Reedijk	43	C^5-thymidine	
McLaughlin	44	replaces nucleotide	

Netzel *et al.*[23] also employ a combination of solid-phase and post-modification chemistries to label oligodeoxynucleotides with $Ru(bpy)_3^{2+}$derivatives. Similar to the procedure described by Dervan, a linker terminating in a primary amine is attached to a cytidine or thymidine base. An oligodeoxynucleotide containing this amino functionality is synthesized and subsequently isolated by HPLC. This primary amine then reacts with the N-hydroxysuccinimide ester of 4-carboxy-4′-methyl-2,2′-bipyridine in solution to form the bipyridine-labeled oligodeoxynucleotides (40–60% yields), which are purified by HPLC. Next, $Ru(bpy)_2(H_2O)_2^{2+}$ and the bpy-labeled oligodeoxynucleotide are coupled in solution to yield the $Ru(bpy)_3^{2+}$-labeled oligodeoxynucleotide. Melting profiles of the HPLC-purified octamers indicate that the Ru-derivatized thymidine results in a more stable duplex than the analogous Ru-labeled cytidine; melting profiles of Ru-labeled thymidine strands are comparable to unmodified duplexes.

Barton *et al.*[38–40] report the solution-phase synthesis of several tethered Ru(II), Os(II) and Rh(III) intercalators. First, an alkylamino linker is coupled to the 5′-terminal ribose of the solid-support-bound oligodeoxynucleotide. Once the oligodeoxynucleotide is cleaved from the solid

support, a metal complex ($[M(phen)(bpy')(Me_2dppz)]^{2+}$ (M=Ru or Os); $Rh(phi)_2(phen')]^{3+}$; or $Ru(phen')dppz)]^{2+}$) containing a carboxylic acid functionality is coupled to the amino functionality in the presence of DCC and HOBt, with low yields. The metallo-oligodeoxynucleotides are purified by HPLC and metal content is quantified by UV-vis spectroscopy.

Oligodeoxynucleotides labeled with europium are reported by Sessler and coworkers.[14] The first step in this protocol requires the synthesis of a 20-mer DNA sequence containing an alkylamine group at either C^5 of an internal thymidine residue or the 5′-terminal phosphate. Reaction of a carboxylic acid-functionalized europium(III) texaphyrin (EuTx) complex with carbodiimide and *N*-hydroxysuccinimide produces the activated ester, which adds directly to the amino-labeled oligodeoxynucleotide in solution. The resulting DNA-EuTx conjugates are purified by electrophoresis.

Bashkin and coworkers[18] report the synthesis of metallo-oligodeoxynucleotides using solution-phase oligodeoxynucleotide chemistry. A terpyridine derivative possessing a free amine is coupled directly to 5-propanoic acid-2′-deoxyuridine using ethyl[3-(dimethylamino)propyl]carbodiimide hydrochloride. The functionalized uridine derivative is then prepared for automated DNA synthesis by reacting the 5′- and 3′-hydroxyl residues with DMT-Cl and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, respectively. This modified nucleobase phosphoramidite is subsequently incorporated in an oligodeoxynucleotide. After hybridization, copper (II) is added to a DNA·RNA duplex to promote hydrolytic cleavage of RNA.

Meade and Kayyem[41] describe a procedure for attaching a ruthenium complex to the 2′-terminal position of an oligodeoxynucleotide. Using standard solid-phase phosphoramidite chemistry, a 2′-amino-uridine (5′-DMT-2′-*N*-trifluoroacetylamino-2′-deoxyuridine phosphoramidite) is incorporated as the 5′-terminal base in an oligodeoxynucleotide (octamer). This HPLC-purified 2′-amino oligodeoxynucleotide is then annealed with its complementary sequence for subsequent metal complex binding, induced by the addition of a solution of $[Ru(bpy)_2CO_3]$ (followed by imidazole) or $[Ru(NH_3)_4(py)]^{2+}$ to the duplex solution under an inert atmosphere. Denaturation of the resulting modified duplex in 7 M urea followed by HPLC purification affords the Ru-labeled single strand in yields of 25–50%. Melting temperatures for

singly-modified duplexes are approximately 8 °C lower than unmodified duplexes.

The synthesis of oligodeoxynucleotides labeled with the electrochemically active metal complex, ferrocene, is reported by Ihara and coworkers.[21] Aminohexane is first attached to the 5′-terminus of the oligodeoxynucleotide using 6-(*N*-trifluoroacetylamino)-hexyl-β-cyanoethyl-*N,N*-diisopropylphosphoramidite and a standard solid-phase DNA synthesis protocol. After deprotection and purification, this amino-terminated oligodeoxynucleotide couples to the activated ester of ferrocene carboxylic acid in solution. HPLC purification affords the labeled oligodeoxynucleotide in approximately 70% yield. The ferrocene-labeled oligodeoxynucleotide is detected electrochemically after hybridization to a complementary probe strand that is attached to a gold electrode.

Similarly, Ortmans *et al.*[42] incorporate a 5-(3-*N*-(9-FMOC)-aminopropyl)-2′-deoxyuridine via automated DNA synthesis. The synthesized amino-labeled oligodeoxynucleotide is cleaved from the column and completely deprotected. The activated ester of $[Ru(tap)_2(dip)]^{2+}$, formed by the addition of N,N,N′,N′-tetramethyl(succinimido)urondium tetrafluoroborate to the carboxylic acid functionality, then reacts with the oligodeoxynucleotide to give the Ru-labeled product in 20% yield. Gel electrophoresis affords the purified oligodeoxynucleotide, and electrospray MS confirms attachment of the ruthenium complex to the oligodeoxynucleotide. The melting temperatures of the mono-labeled duplexes are comparable to the unlabeled duplexes (ΔT_m ~2° C).

Reedijk *et al*[43] report a novel synthetic route to site-specifically platinated oligodeoxynucleotides. In this procedure, the pentamer 5′-TTGGT-3′ is synthesized, and then cleaved from the column, leaving only the isobutyryl groups at the N^2-position of the guanines intact. Addition of $[Pt(en)Cl(H_2O)]^+$to the base-protected pentamer, followed by removal of the isobutyryl groups by aminolysis, yields the Pt-labeled oligodeoxynucleotides with nearly quantitative labeling efficiency. A guanosine nucleotide containing a new O^6-protecting group (diphenylcarbamoyl) retards platination at undesired guanines in the oligodeoxynucleotide strand.

Wiederholt and McLaughlin[44] incorporate a modified bipyridine phosphoramidite in place of a nucleoside during normal DNA synthesis. After cleavage from the column and purification, the bpy-oligodeoxynucleotide is refluxed with *cis*-dichloro-bis-2,2′-bipyridine ruthenium (II) dihydrate.

Midway through the reaction, pyridine is added to the mixture to remove non-specifically bound ruthenium from the N^7 of guanine. The ruthenium-DNA conjugates are then purified on a Sephadex G-25 column. Gel-shift assays, PAGE, FPLC, UV-vis spectroscopy and fluorescence spectroscopy are used to characterize the product. The T_m of the Ru-labeled DNA is 17 °C lower than that the unmodified duplex.

SOLID-PHASE LABELING

While solution-phase couplings of metallo groups to different moieties of the oligodeoxynucleotide are successful and sufficiently high-yielding for the purposes of characterization and some studies, these synthetic methods are often hampered by low yields, side reactions and multiple purification steps. More recent attempts to synthesize metallo-labeled oligodeoxynucleotides focus on incorporation of the metal or metal complex on solid support. There are several advantages to a solid-phase synthetic approach, including fewer side reactions, higher yields, and reduced purification steps. Solid-phase methods are also used to label oligodeoxynucleotides with more than one type of metal complex. Importantly, the current advantages of solid-phase chemistry can be combined with automated DNA synthetic techniques to afford a virtually "hands-off" procedure to synthesize site-specifically metallo-labeled DNA.

There are two approaches to solid-phase coupling of metal complexes. The first method incorporates a functional group at the oligodeoxynucleotide (at the ribose, nucleobase or phosphate) for subsequent coupling to a metal complex while the oligodeoxynucleotide is still bound to the support (*on-column derivatization*). The second approach is to incorporate a nucleoside or phosphoramidite to which the metal group is already bound (*metallo-phosphoramidite or metallo-H-phosphonate*).

On-Column Derivatization

The syntheses of metallointercalators tethered to oligodeoxynucleotides at the 5′-terminus using an *on-column derivatization* method are reported by Barton and coworkers.[45–48] As shown in Figure 2, an alkylamino linker is coupled to the 5′-terminal ribose of the oligodeoxynucleotide while still

bound to the CPG support in two steps.[49] First, the 5′-hydroxyl is activated with carbonyldiimidazole (a); then the activated 5′-terminus reacts with 1, 9-diaminononane (b) to give the 5′-amino-labeled oligodeoxynucleotide. Next, the N-succinimidyl ester of a metal complex ($Rh(phi)^2bpy'^{3+}$ or $Ru(phen)(bpy')(dppz)^{2+}$) and excess DIPEA are added (c) to the amino-functionalized oligodeoxynucleotide on solid support (CPG). After 12 hours, the solid support is washed with ethanol and dichloromethane. The metallo-labeled oligodeoxynucleotides are subsequently cleaved from the resin (d) and purified by HPLC. Characterization of the metallo-oligodeoxynucleotides is done by UV-vis spectroscopy, MS, and enzymatic digestion and base analysis. Melting temperatures (T_m) of the labeled strands are slightly higher (5 °C) than unlabeled duplexes.

Grinstaff and coworkers[50,51] report a facile procedure for the site-specific modification of oligodeoxynucleotides during automated solid-phase synthesis that utilizes both solid-phase DNA and Pd(0) cross-coupling chemistries. This Pd(0) ***on-column derivatization*** procedure allows for the incorporation of a metal complex at both terminal and internal nucleobase positions in the oligodeoxynucleotide. As outlined in Figure 3, normal DNA synthesis is performed until incorporation of 5′-DMT-3′-cyanoethyl-*N,N*′-diisopropyl phosphoramidite-2′-deoxy-5-iodouridine (a). At this stage, automated synthesis is paused, before removal of the 5′-DMT protecting group leaving the oligodeoxynucleotide on the resin. The column is then removed from the synthesizer and sparged with N_2. Next, the alkyne-modified ruthenium tris-bpy complex is added to the column (b), as well as the Pd(0)/CuI catalyst and solvent (dry DMF/TEA), and placed on a shaker. After 3 hours, the column is rinsed with DMF/TEA and acetonitrile and then dried with N_2. The column is replaced on the synthesizer and DNA synthesis is resumed (c) with additional nucleotides being incoporated. After synthesis the metallo oligodeoxynucleotides are cleaved from the column and deprotected according to the standard procedures (d). HPLC purification affords ruthenium-labeled oligodeoxynucleotides of varying sequences, ranging from four to sixteen nucleotides long. Collection and analysis of the DMT fractions during automated synthesis showed efficient phosphoramidite coupling of both the standard pyrimidine and purine nucleosides, as well as with 5-iodouridine, throughout the procedure (>95%). Analysis of the HPLC traces of the crude oligodeoxynucleotide products indicate Pd(0)

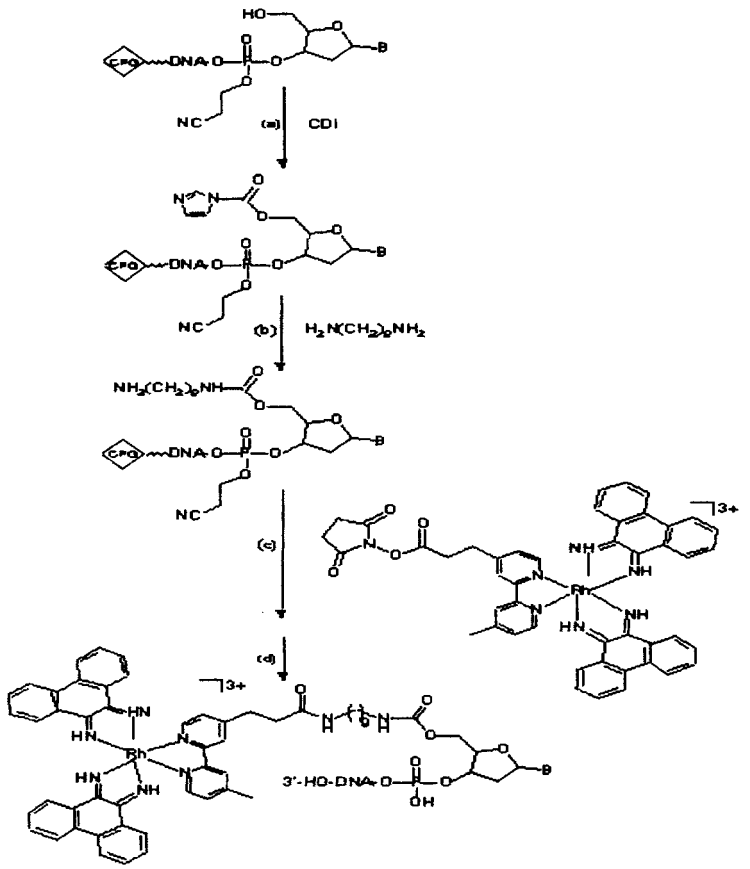


FIGURE 2 On-column derivatization

cross-coupling efficiencies from 75 to 90%. These ruthenium-labeled oligodeoxynucleotides are characterized by UV-vis spectroscopy, emission spectroscopy, CD spectroscopy, mass spectrometry, enzymatic digestion and melting profiles.

This coupling procedure is applicable to other compounds as well; for example the site-specific labeling of oligodeoxynucleotides with ferrocene is also described by Grinstaff and Beilstein.[52] Using a similar oligodeoxynucleotide synthetic strategy and an alkyne-modified fer-

rocene derivative, ferrocene is introduced at the C^5-position of uridine, with coupling yields of 50%. The ferrocene-labeled strands form stable duplexes with T_m differences of 4°C or less. CD spectra also confirm that the labeled duplexes retain B-form DNA structure.

Metallo-Phosphoramidite and Metallo-H-Phosphonate

Although solid-phase and on-column derivatization methods are worthwhile in terms of yield and product purity, there are limitations to their utility. To synthesize relatively large quantities of derivatized oligodeoxynucleotides, preparation of an actual nucleoside or nucleoside analogue phosphoramidite can be advantageous. As mentioned previously in the automated solid-phase DNA synthesis section, nucleoside phosphoramidites are activated with tetrazole and coupled in extremely high yield to a deprotected 5′-hydroxyl on the growing oligodeoxynucleotide strand (3′- to 5′-direction). Currently, several groups are taking advantage of this efficient coupling chemistry and are synthesizing a variety of metallo-labeled oligodeoxynucleotides by using metal-derivatized phosphoramidites in a standard automated DNA cycle. In addition to being compatible with the reagents of the automated synthesis cycle, two additional criteria must be met. The label must be amenable to phosphorous(III) chemistry and possess an unprotected hydroxyl group. Reacting this hydroxyl with either (β-cyanoethoxy)-bis-diisopropylaminophosphine [method A]/tetrazole or (β-cyanoethyl)-diisopropylaminochlorophosphoramidite/DIPEA [method B], as shown in Figure 4, yields the metallo-phosphoramidite. Automated solid-phase synthesis then follows, as shown in Figure 1. If the phosphoramidite also contains a 5′-DMT-protected hydroxyl, additional bases can then be added, otherwise the chain is terminated. Upon completion of synthesis, standard deprotection followed by purification affords the labeled oligodeoxynucleotides, typically in good to high yields. As described in Tables II and III, the phosphoramidite method is successful for labeling DNA with iron, ruthenium, and osmium complexes. Additionally, platinum complexes are incorporated using the H-phosphonate strategy, a related phosphorous(III) coupling approach.[36] Overall, these methods are general and compatible with a variety of metals and functional groups with labels successfully incorporated at various positions in an oligodeoxynucleotide.

Lippard *et. al.*[12] report the labeling of the N^7-position of guanosine with platinum(II). Phosphoramidite chemistry is not compatible with platina-

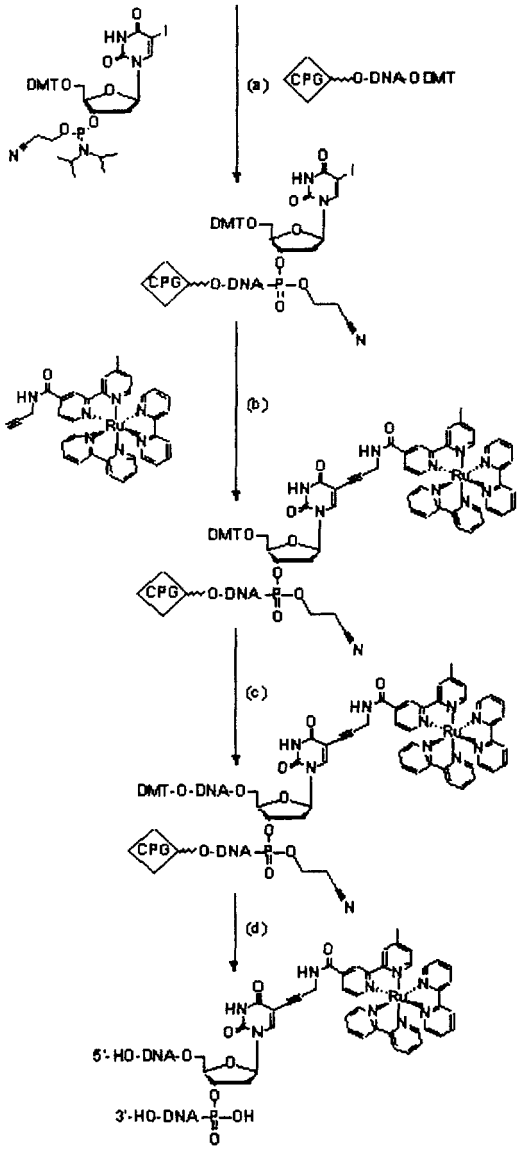


FIGURE 3 Pd(0)/CuI-catalyzed coupling for the on-column derivatization of oligodeoxynucleotides with metal complexes

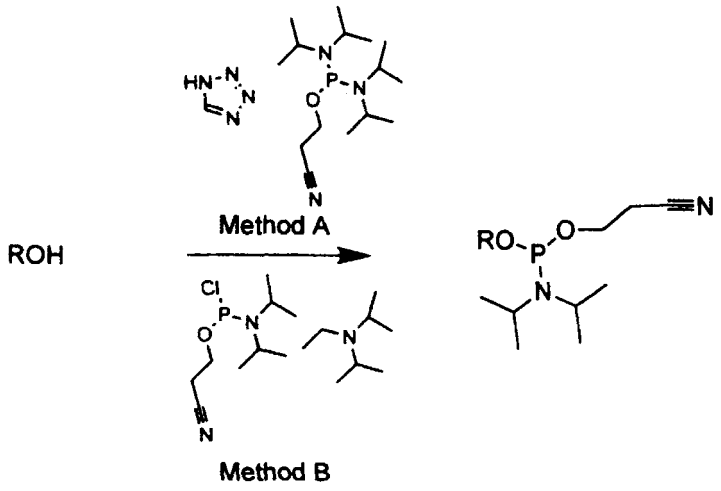
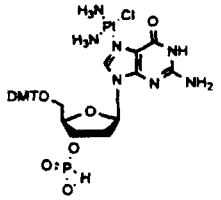
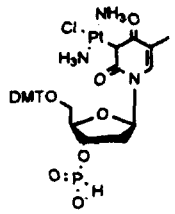
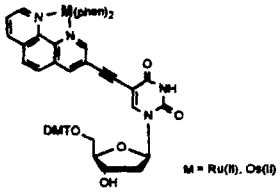
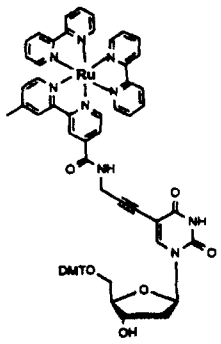


FIGURE 4 General reaction for phosphoramidite formation

tion of guanosine. However, the H-phosphonate coupling strategy is employed with success for the synthesis of platinated oligodeoxynucleotides. Treatment of the DMT-protected H-phosphonate of guanosine with *cis*-$[Pt(NH_3)_2(DMF)Cl]NO_3$ yields the platinated guanosine derivative. Manual coupling of this H-phosphonate with pivaloyl chloride in 2-picoline attaches the guanosine platinum complex to the growing oligodeoxynucleotide. This compound is sufficiently stable to automated synthesis conditions, and deprotection in concentrated NH_3(aq)/triethylamine and HPLC purification affords the site-specifically platinated DNA.

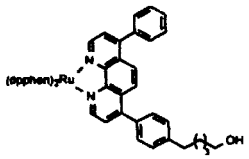
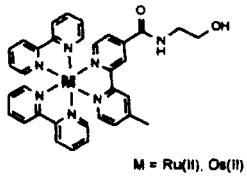
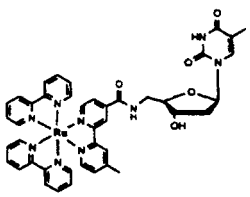
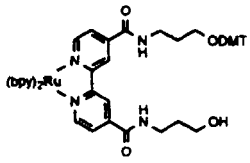
Introduction of Pt(II) at the N^3-position of thymidine and use of this modified nucleotide in automated DNA synthesis is also described by Cech *et al.*[13] DMT-protected thymidine H-phosphonate is treated with KOH to deprotonate the N^3-position. Reaction of this salt with *trans*-$[(NH)_3PtCl_2]$ yields the platinated thymidine H-phosphonate for use in automated synthesis. A manual coupling is used and additional nucleotides are added using conventional automated synthesis. However, numerous side reactions and deplatination of the oligodeoxynucleotide result in low yields. Purification of platinated oligodeoxynucleotides, followed by enzymatic digestion, HPLC, and ion spray MS confirm the platinated nucleotide to be *trans*-$[(NH_3)_2(py)(N^3\text{-thymidine})Pt]^+$.

TABLE II Metallo-nucleobase H-phosphonates and phosphoramidite precursors

Metal Complex	*Research Group*	*Phosphoramidite Synthesis*[a]	*Coupling*	*Ref.*
	Lippard *et al.*	H-phosphonate	Manual	12
	Cech *et al.*	H-phosphonate	Manual	13
	Tor and Hurley	Method B	Manual	53
	Grinstaff *et al.*	Method B	Automated	54, 55

a. See Figure 4.

TABLE III 5′-Terminus metallo-phosphoramidite precursors

Metal Complex	*Research Group*	*Phosphoramidite Synthesis*[a]	*Coupling*	*Ref.*
	Letsinger *et al.*	Method B	Manual	56
	Bannwarth and Schmidt	Method A	Manual	22, 57
 M = Ru(II), Os(II)	Grinstaff *et al.*	Method B	Automated	58
	Grinstaff *et al.*	Method B	Automated	59
	Lewis *et al.*	Method B	Manual	60

a. See Figure 4.

Site-specific modification of an oligodeoxynucleotide at the nucleobase with ruthenium complexes is described by the groups of Tor and Grinstaff. Both strategies include a Pd(0)/Cu(I) Sonagashira coupling as a key step to form a C-C bond between a terminal alkyne and an

aryl/alkenyl halide. Tor and Hurley[53] use the Sonagashira reaction to couple a 5-ethynyl derivatized 5′-DMT-uridine with [M(phen)$_2$(3-bromo-1,10-phenanthroline)](PF$_6$)$_2$ [M=Ru(II), Os(II)] to afford the metallo-nucleoside. Subsequent transformation to the metallo-nucleoside phosphoramidite and incorporation in an oligodeoxynucleotide is performed manually. Site-specifically labeled metallo-oligodeoxynucleotides are obtained in good yields after HPLC. Thermal denaturation studies show the unlabeled duplex to be destabilized by up to 3 °C, depending on the position of the metal complex.

Grinstaff *et al.*[54,55] report the synthesis of a ruthenium(II) metallo-nucleoside phosphoramidite for automated DNA synthesis. An alkyne-derivatized ligand bipyridine, N-(2-propynyl)-4-methyl-2,2′-bipyridine-4-carboxamide, is heated at reflux with Ru(bpy)$_2$Cl$_2$ to afford Ru(bpy)(pmbc)(PF$_6$)$_2$. This terminal alkyne complex couples with 3′,5′-dibenzoyl-5-iodo-2-deoxyuridine to afford the ruthenium-nucleoside. Removal of the benzoyl groups with NH_3, followed by protection of the 5′-hydroxyl with a DMT group yields the protected metallo-nucleoside. Phosphoramidite formation, followed by incorporation via automated DNA synthesis yields site-specifically labeled metallo-oligodeoxynucleotides. Labeling is possible at any position and the synthesis of both short and long oligodeoxynucleotides is reported. Thermal denaturation experiments show no substantial perturbation (ΔT_m<1 °C) from unlabeled duplexes and CD spectra confirm that the B-DNA helical form is preserved.

Incorporation of an iron complex by Letsinger *et al.*[56] involves terminal labeling of an oligodeoxynucleotide strand with a derivatized ferrocene. The phosphoramidite of 6-hydroxyhexylferrocene is coupled with the last base in the synthesis cycle. The ferrocene "caps" the 5′-terminus of the oligodeoxynucleotide. Melting temperatures for the labeled (42 °C) and unlabeled oligodeoxynucleotide (41 °C) demonstrate that there is no dramatic perturbation of the duplex. In addition, the incorporation of a thiol linker at the 3′-position in the beginning of automated synthesis allows for subsequent deposition to an electrode surface. Reversible electrochemistry is observed with the ferrocene-labeled oligodeoxynucleotide, consistent with the parent ferrocene compound.

The phosphoramidite approach for labeling DNA with ruthenium(II) complexes is first described by Bannwarth and Schmidt.[22,57] A Ru(dpphen)$_3^{2+}$ complex is synthesized containing one primary

hydroxyl group at the para-position on one of the appended phenyl rings. The corresponding ruthenium complex phosphoramidite is then manually coupled to an oligodeoxynucleotide, "capping" the oligodeoxynucleotide strand at the 5′-terminus.

Grinstaff *et al.*[58] describe the use of a ruthenium(II) tris-diimine phosphoramidite to label the 5′-terminus in a fully automated fashion. A hydroxy-functionalized bipyridine ligand, N-(2-hydroxyethyl)-4-methyl-2,2′-bipyridine-4-carboxamide, is heated at reflux with $Ru(bpy)_2Cl_2$ to afford $Ru(bpy)(hmbc)(PF_6)_2$. This complex readily forms a phosphoramidite in the presence of β-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite to yield the complex for automated DNA synthesis. Good yields of $Ru(diimine)_3$-modified oligodeoxynucleotides are obtained after HPLC purification (>75%). Thermal denaturation experiments show that these 5′-terminally labeled oligodeoxynucleotides are similar to the unlabeled duplex (ΔT_m of less than 2 °C) and that the ruthenium complex does not significantly alter the duplex strand.

Recently, the labeling of DNA at the 5′-aminoribose of an oligodeoxynucleotide is described by Grinstaff *et al.*[59] A $Ru(diimine)_3^{2+}$-thymidine derivative phosphoramidite based on 5′-[$Ru(bpy)_2$(4-methyl-2,2′-bipyridine-4′-carboxamido)]$^{2+}$-thymidine, is synthesized in five steps starting from thymidine. Incorporation at the 5′-terminal during automated DNA synthesis yields 5′-ribose ruthenium labeled oligodeoxynucleotides. Thermal denaturation experiments on respective unlabeled and $Ru(diimine)_3^{2+}$ labeled 13-mer duplexes are similar. A decrease of 3 °C in the melting temperature is observed, and this small change indicates that labeling the 5′-terminal nucleotide does not dramatically alter the oligodeoxynucleotide duplex structure. Circular dichroism experiments further support this observation. CD spectra of the unlabeled and $Ru(diimine)_3^{2+}$ labeled oligodeoxynucleotide display the spectral features for B-DNA.

Lewis *et al.*[60] are able to bridge two complementary strands of DNA using a difunctional ruthenium(II) complex to make hairpin structures. A 2,2′-bipyridine-4,4′-dicarboxylic acid is converted to its analogous bis-(N-(3-hydroxypropyl))arenedicarboxamide. Next, one hydroxyl is protected with a DMT group, and then this ligand is treated with $Ru(bpy)_2Cl_2$ to afford the $Ru(bpy)_2$(DMT-dabp)$(PF_6)_2$ complex. Finally, the second hydroxyl is converted to the phosphoramidite for oligodeoxynucleotide synthesis. In a glove box, a manual coupling with

tetrazole attaches the metal complex to the 5′-hydroxyl of the oligodeoxynucleotide. The DMT group on the complex is then cleaved, and automated synthesis resumes to complete the hairpin. Thermal denaturation studies are consistent with a hairpin-type structure.

In summary, significant progress in the synthesis of site-specifically labeled metallo-oligodeoxynucleotides is described in the recent literature. Labeling procedures are reported for modifying oligodeoxynucleotides at the ribose, phosphate, or nucleobase moieties. Of particular significance is the successful use of solid-phase methods and automation to synthesize metallo-oligodeoxynucleotides, reducing the labor and time required to synthesize sufficient quantities of material for biochemical and biophysical studies.

While great strides have been accomplished, certain limitations still exist. For example, labeling the nucleobase with metal complexes is mostly limited to thymine: labeling the other three nucleosides (adenosine, cytidine, and guanosine) will expand the current repertoire available for study. With respect to the metal complexes employed, the majority of work reported describes the incorporation of d^6, d^8, or d^{10} metal complexes. New procedures are needed that tolerate other classes of metal complexes. No reports of site-specifically metallo-labeled RNA are described. Expanding these synthetic methods to include RNA labeling would allow further study of RNA and RNA-DNA interactions. More detailed characterizations are also needed to ascertain the structure after metallo-labeling. Advances in both NMR spectroscopy and X-ray crystallographic techniques in the last five years should allow for these studies to be performed.

With regard to the applicability of metallo-oligodeoxynucleotides for DNA diagnostics and gene recognition, new transition metal probes are needed. For example, environmentally sensitive probes that recognize specific nucleic acid sequences or local microstructures will aid in delineating nucleic acid structure/conformation and reactivity. New labels for probing the mechanism(s) of DNA-mediated charge transfer are also in demand, and metal complexes provide a unique means to easily assess the driving force (ΔG) and reorganization energy (λ) dependencies for these reactions. Finally, the development and use of micro- or nano-sized devices for detecting DNA either spectroscopically or electrochemically require metallo-oligodeoxynucleotides, to be amenable to fabrication procedures and techniques.

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