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Synthesis and characterization of oligonucleotide conjugates bearing electroactive labels

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ABSTRACT

Oxime bond formation has been applied to the preparation of oligonucleotides labeled with electrochemical ferrocene and viologen labels. Aminooxy functionalized ferrocene and viologen derivatives were prepared by a straightforward route and efficiently conjugated with aldehyde containing oligonucleotides either at 3′ or 5′ end. Both labels were found to not disturb the recognition properties of the oligonucleotide. The versatility of the method was further demonstrated by preparing bi-functionalized conjugates with a disulfide at 3′ end and an electrochemical label at 5′ end.

Oligonucleotide based biosensors[1] play an important role in diagnosis since they are used for detecting a large panel of analytes including nucleic acids,[2] proteins,[3] small organic molecules,[4] metals[5] as well as whole cells.[6] For these reasons, a large number of research projects are devoted to the development of DNA-based biosensors for which the transduction of the recognition event is performed through optical,[7] gravimetric[8] or electrochemical methods.[9] The latter provide high sensitivity and have the advantage of needing only simple and compact apparatus.

One strategy for electrochemical monitoring of biomolecular recognition is the comparison of the signal due to an electroactive label chemically linked to the oligodeoxyribonucleotide (ODN) in absence and in presence of the target. The most studied redox label is certainly ferrocene (Fc) since this molecule allows synthetic modifications thanks to the high chemical stability of the iron II η^5 complex. Moreover, the value of the oxidation potential is not in the range of the O_2 reduction and cyclovoltammetry (CV) can be performed without further precautions. Preparations of ferrocene–ODN conjugates have been described[10] including direct incorporation of a Fc phosphoramidite during ODN synthesis,[11] amidative oxidation of H-phosphonate linkage[12] or enzymatic incorporation of a Fc nucleotide triphosphate.[13] Fragment coupling strategy,[14] that is, chemoselective coupling of reporter and ODN bearing mutually-reactive functional moieties, is of great interest because each fragment can be separately prepared with the most efficient chemistry available. Up to date, only few applications of this strategy for ferrocene labeling of ODN have been reported such as the coupling of a ferrocenyl activated ester with an amino modified ODN,[15] or a CuAAC (copper catalyzed alkyne cycloaddition) reaction between oligonucleotide bearing an alkyne group and an azido-modified Fc.[16]

Viologens (1,1′-disubstituted 4,4′-bipyridinium dications) represent another class of redox active molecules, showing low redox potentials[17] far from those of nucleobases and thus are excellent candidates for the development of electrochemical nucleic acids biosensors. However, viologen cannot be directly incorporated during the automated DNA synthesis because of incompatible reactivities. Indeed degradation of viologen can occur during ammonia treatment required for deprotecting ODNs.[18] So far there is only one report describing conjugation of viologen with oligonucleotides in solution, the method involving the reaction between an activated ester of a methyl viologen derivative and an amino containing ODN.[19] This strategy showed certain drawbacks because the coupling reaction involves carbodiimide activated intermediate, which is usually unstable in aqueous medium. This could be overcome by using a large excess of activated ester of viologen.

With the aim of developing an efficient and reliable strategy for synthesizing oligonucleotide conjugates with either ferrocene (Fc) or methyl viologen (MV) labels, we focused on the use of oxime bond formation. This approach has been proved to be efficient

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for ODN conjugation with various reporters including peptides, carbohydrates or fluorescent labels.[20] In the context of electrochemical labeling of ODNs the strategy requires the preparation of ODN carrying an aldehyde group for subsequent coupling reaction with aminooxy containing redox labels.[21] Herein we report on the preparation of aminooxy ferrocene **1**[22] and aminooxy viologen **2** (Fig. 1) and the subsequent coupling reaction with aldehyde containing oligonucleotides either at 3′ and 5′ end. The hybridization and the electrochemical properties of the conjugates are also described.

Protected precursor **5** of aminooxy ferrocene **1** was synthesized in three steps starting from *N*-(5-Hydroxy-3-oxapentyl)ferrocenoylamide **3** (Scheme 1).[23] The hydroxy group was converted into tosylate **4**, which was substituted with ethyl acetohydroxamate under phase transfer catalysis (PTC) conditions leading to aminooxy-protected ferrocene **5** with an overall yield of 72% from **3**. PTC was used for preventing β-elimination of the tosylate group under the basic conditions required for substitution.[24]

Precursor **9** of aminooxy-methyl-viologen **2** was synthesized according to the following procedure (Scheme 2). Trityl protected aminooxy alcohol **6**[25] was first converted to mesylate **7**, which was used for preparing the monocation **8** through nucleophilic substitution of mesylate group by 4,4′-bipyridine; the methane sulfonate counter ion was then exchanged with iodide. Addition of iodomethane led to trityl protected aminooxy viologen **9**.

The aminooxy protecting groups were kept for storage and were removed just before the coupling reaction with ODNs. Free aminooxy compounds **1** and **2** were obtained in quantitative yield after treatment of **5** and **9**, respectively, with a 45/45/5 (v/v/v) CH_3CN/H_2O/TFA solution and freeze drying. Compounds **1** and **2** were used for ODN conjugation without further purification.

The efficiency and versatility of the conjugation were first evaluated on model undecamers bearing an aldehyde group either at the 3′ end (5′CGC ACA CAC GC X3′ **10**, with X = CHO) or at the 5′ end (5′Y CGC ACA CAC GC3′ **11**, with Y = CHO). These ODNs were synthesized according to previously reported methods (see the Supplementary data).[26] The conjugation reactions were carried out in ammonium acetate buffer (0.4 M, pH 4.6) by using a slight excess of aminooxy derivatives **1** or **2** (Scheme 3). The course of the reaction was followed by reverse phase HPLC and the reaction proceeded essentially to completion within 6–8 h to yield the conjugates **10a**–**b** and **11a**–**b** as depicted in Scheme 3. The conjugates were almost obtained with satisfactory yields after RP-HPLC purification.[27]

Crude reaction mixtures were found cleaner when the conjugation was performed at the 5′ end (**11a** and **11b**) rather than at the 3′ end (**10a** and **10b**). This could be due to the transposition of the 3′ diol during ODN synthesis leading to loss of the diol group via dephosphorylation.[28] The conjugates **10a**,**b** and **11a**,**b** were characterized by ESI-MS. In all cases, the experimentally determined molecular weights were in excellent agreement with the calculated values (Table 1).

The hybridization properties of conjugates **10a**,**b** and **11a**,**b** were investigated by thermal denaturation and circular dichroism experiments. This study suggests that the redox labels have neither destabilizing nor perturbation effects on the conformation properties of the ODN recognition process (see the Supplementary data).

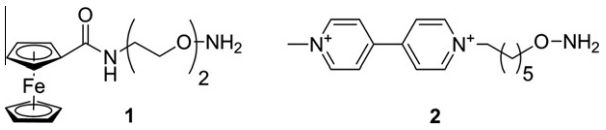
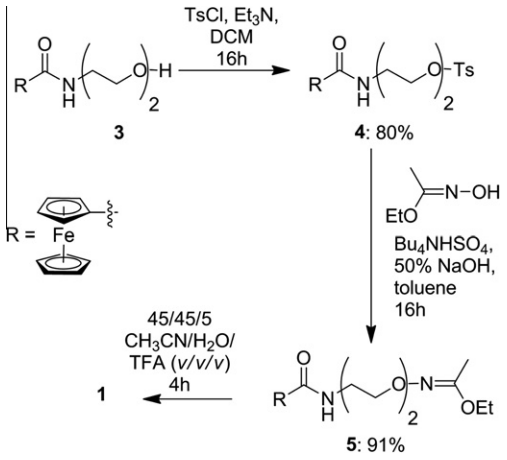
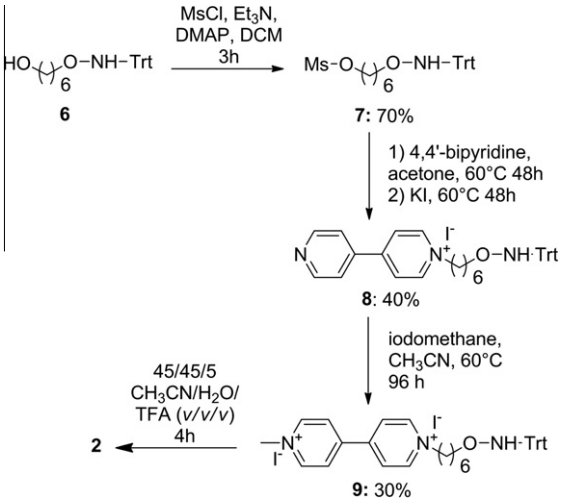


Figure 1. Structures of *N*-[5-aminooxy-3-oxapentyl] ferrocenoylamide **1** and 1-[6-aminooxyhexyl]-1′-methyl-4,4′-bipyridinium **2**.



Scheme 1. Synthesis of *N*-[5-aminooxy-3-oxapentyl] ferrocenoylamide **1**.

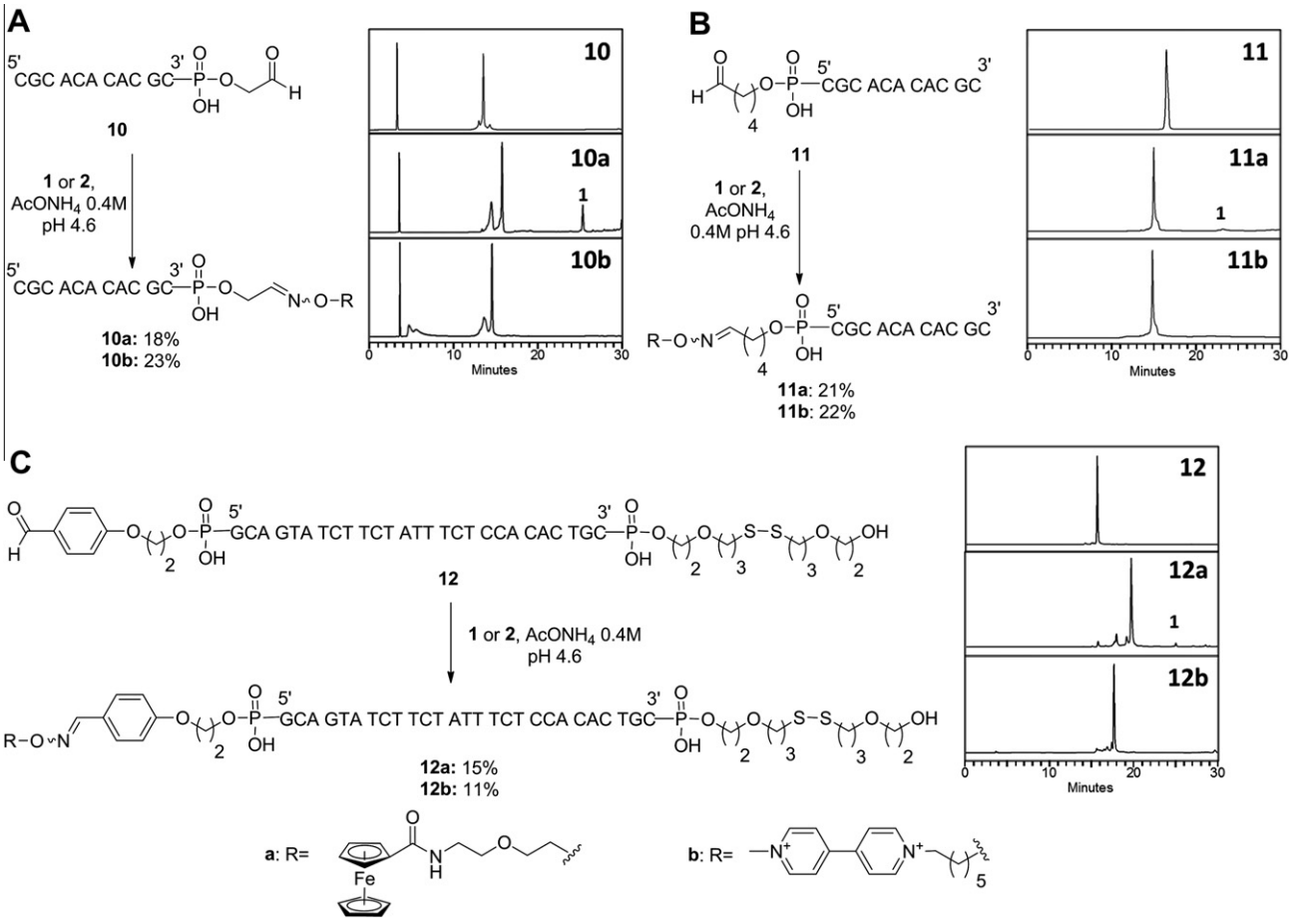


Scheme 2. Synthesis of 1-[6-aminooxyhexyl]-1′-methyl-4,4′-bipyridinium **2**.

For demonstrating that the method might be useful for the elaboration of electrochemical biosensors, the compatibility with a 3′-disulfide moiety on oligonucleotides was studied. Indeed, disulfide is the precursor of thiol function commonly used for anchoring oligonucleotides on gold surfaces.[29] A model 3′-disulfide 5′-benzaldehyde[30] ODN was synthesized (5′X GCA GTA TCT TCT ATT TCT CCA CAC TGC Y3′, X = 5′-benzaldehyde, Y = 3′-disulfide, **12**). Benzaldehyde moiety was chosen instead of 1,2-diol because of the instability of the disulfide linkage upon periodate treatment required for generating aldehyde from diol.[31] This ODN was conjugated with the two aminooxy electrochemical labels **1** and **2** to obtain conjugates **12a** and **12b**, respectively (Scheme 3, C).

RP-HPLC profiles showed almost quantitative conjugation reaction (Scheme 3, C) and the conjugates **12a**,**b** were purified by RP-HPLC[27] and unambiguously characterized by ESI-MS (Table 1).

In order to demonstrate that the electrochemical properties of the labels were not affected, CV of conjugates **12a** and **12b** were recorded in a HEPES buffer containing 1M of sodium perchlorate on screen-printed carbon electrodes (Fig. 2). Conjugate **12a** showed a reversible redox signal ($E_{1/2}$ = 0.4 V vs Ag/AgCl) consistent with the ferrocene bearing an amido group. The CV of conjugate **12b** was recorded under inert atmosphere in order to avoid oxygen reduction and showed two reversible one-electron reduction peaks, one at −0.47 V (vs Ag/AgCl) and one at −0.74V (vs Ag/AgCl) consistent with the presence of the viologen.



Scheme 3. Syntheses of conjugates (A: **10a**,**b**, B: **11a**,**b**, C: **12a**,**b**) and RP-HPLC (260 nm) profiles of unlabeled ODN and of crude conjugates.

Table 1
ESI-MS data for conjugates[a]

Conjugate	*m*/*z* Calcd	*m*/*z* Found
10a	3707.3	3707.1
10b	3660.8	3659.2
11a	3747.7	3749.0
11b	3702.8	3701.5
12a	9010.9	9011.1
12b	8963.6	8963.8

[a] Analyses were carried out in the negative mode. $CH_3CN/H_2O/Et_3N$ (50:50:2, v/v/v) was used as the eluent at a flow rate of 8 μL min^{-1}.

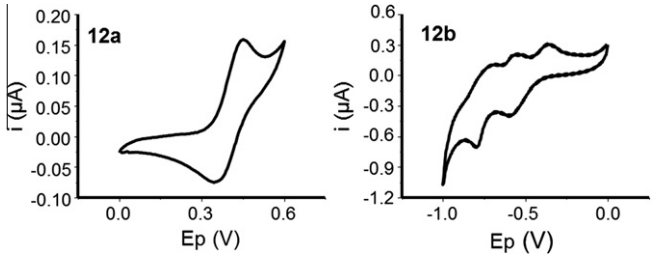


Figure 2. Cyclic voltammograms of conjugates 200 μM **12a**,**b** in HEPES (pH = 7.4) $NaClO_4$ 1 M, scanning rate: 50 mV s^{-1}.

In conclusion, we have developed an efficient method for preparing oligonucleotide conjugates with electrochemical labels with fair isolated yields. Moreover we have also demonstrated that this protocol can be used for preparation of bis functional oligonucleotides with both an electrochemical label and an anchoring function. We believe that this strategy might be useful for elaborating electrochemical nucleic acids biosensors using ferrocene and viologen labels. Indeed both labels possess distinct redox potentials which could be exploited for simultaneous detection of different analytes.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.12.057.

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