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Oligothiophene phosphoramidites for oligonucleotide labelling

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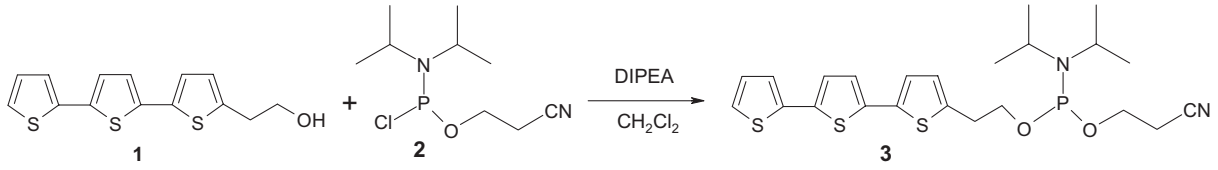
Abstract—We report the synthesis and characterization of two oligonucleotides (a tetramer and a 19-mer) labelled with a fluorescent oligothiophene and obtained by means of the phosphoramidite of the fluorophore. The conjugate compounds were synthesized in solid phase and characterized by means of mass spectrometry, multinuclear NMR, UV–vis and photoluminescence spectroscopies. The results show that this approach is suitable as a general route for the preparation of oligonucleotides labelled with oligothiophene-based fluorophores.

Oligothiophenes are semiconductor and photoactive compounds, which have attracted great attention in recent years.[1–8] Despite their intrinsic fluorescence and the easy tuning of this property via organic synthesis, very little interest has been devoted to the development of fluorescent tags for biomolecules.[9] In this article, we report the easy preparation of a phosphoramidite derived from an oligothiophene and its incorporation in oligonucleotides for use in biochemical studies.

5-(2-Hydroxyethyl)-2,2′:5′,2″-terthiophene[9] **1** (Scheme 1) has a moderate solubility in water where it maintains its photoluminescence with $\lambda_{PL} = 441$ nm when irradiated at optimum wavelength (356 nm). The absorption spectrum does not change when the compound is dissolved alone or when it is mixed with oligonucleotides either in single or double stranded form (not shown), thus demonstrating that it does not intercalate between oligonucleotide bases. Also the absorption spectrum of **1** does not change after heating at 50 °C in the presence of concentrated ammonia, so we assumed that the structure would resist the basic hydrolysis necessary to remove the protecting groups of the traditional supported synthesis of oligonucleotides, and we checked the possibility of introducing this compound as a tag using the phosphoramidite approach in the synthesis of oligonucleotides.[10]

We prepared the phosphoramidite of **1** by the synthesis described in Scheme 1.[11] Briefly, 44 mg of terthiophene **1** were dissolved in 5 mL of dichloromethane and 0.13 mL of DIPEA. 2-Cyanoethyl-*N*,*N*-diisopropyl-chlorophosphite (**2**, 71 mg) was added under anhydrous atmosphere. After 30 min, the reaction mixture was poured in a separation funnel containing dichloromethane and water. After phase separation, the organic phase was dried and concentrated under reduced pressure. Pure **3** was obtained after chromatography on silica gel eluting with CH_2Cl_2/EtOAc/Et_3N 45:45:10 v:v:v.



Scheme 1. Preparation of the phosphoramidite of **1**.

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The suitability of compound **3** to prepare fluorescent oligonucleotides was tested by labelling two different sequences on a 10 μmol scale: a tetramer (T_4), used for a full chemical characterization, and a 19-mer (ACCAC-CCTTCGAACCACAC), to check a possible influence of the sequence length on the fluorescence emission. For the synthesis, compound **3** was dissolved to a final concentration of 0.1 M in acetonitrile and used on a spare position on a commercial synthesizer (Pharmacia Gene Assembler II plus) to introduce the tag at the 5′ position of the oligonucleotide by conventional protocols.[12,13] At the end of the synthesis, the cartridges containing the supported protected oligonucleotides were sealed in vials with concentrated ammonia and deblocked by warming at 50 °C overnight. The oligonucleotides were then purified by reversed phase chromatography exploiting the lipophilicity induced by the tag. The structure of the terthiophene–tetramer conjugate (dye-T_4) is shown in Figure 1 and the corresponding NMR ^{1}H and ^{31}P spectra are reported in Figures 2 and 3.

The proton spectrum (Fig. 2) of this conjugate clearly demonstrates the presence of four protons pertaining to tymidine (H-5, in the range 7.2–7.8 ppm), seven aromatic protons pertaining to the thienyl rings of the dye, (from 7.2 to 6.6 ppm) and of four protons pertaining to sugars (H1′ from 6.3 to 5.9 ppm).

The phosphorus NMR spectrum (Fig. 3) also shows two different peaks attributed to the phosphorus connecting the dye with the oligonucleotide and to those between the sugars, in the expected 1 to 3 ratio. The identity of the conjugated T_4 and 19-mer (dye-19-mer) was also confirmed by mass spectrometry (Table 1).

Both conjugates showed the absorption spectra featuring the oligonucleotide maximum wavelength at

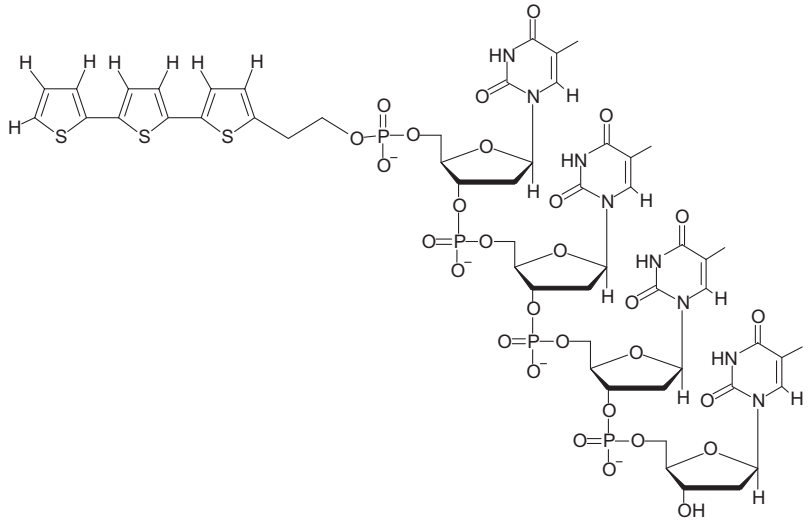

Figure 1. The terthiophene–T_4 conjugate prepared with **3**.

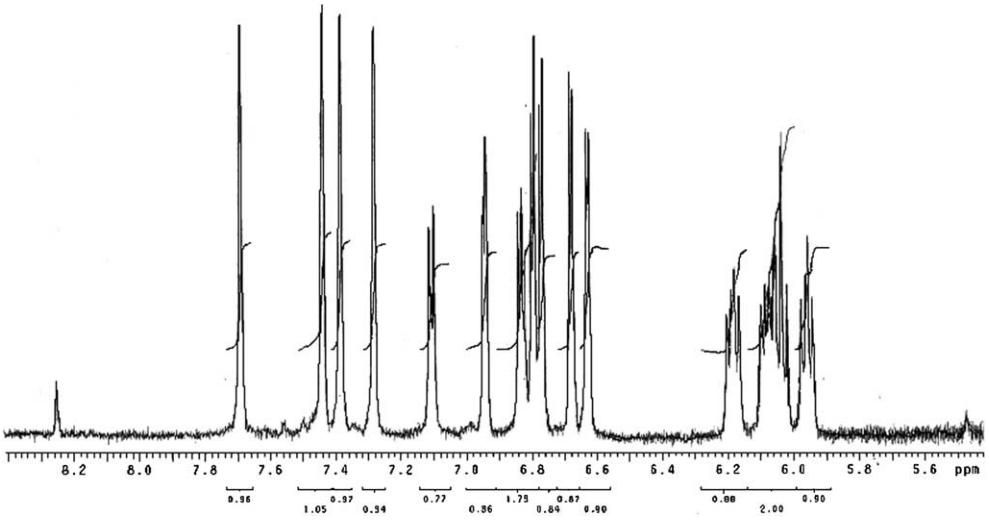

Figure 2. ^{1}H NMR spectrum in D_2O of the terthiophene–T_4 conjugate. From 7.7 to 7.2 ppm H-6 of tymidines (4H), from 7.2 to 6.6 ppm aromatic hydrogens of terthiophene (7H), from 6.2 to 5.9 ppm H1′ of tymidines (4H).

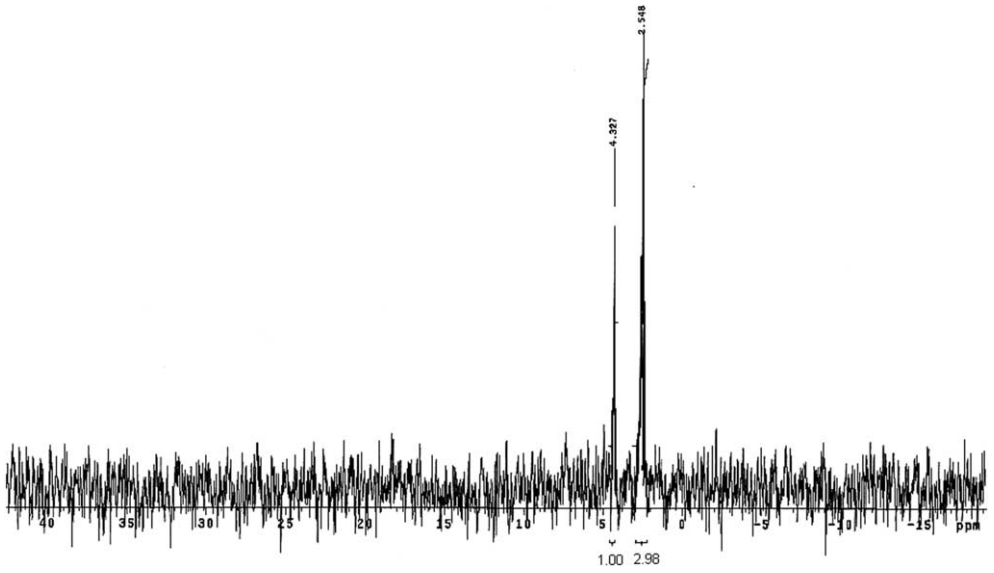

Figure 3. ^{31}P NMR spectrum in D_2O of the terthiophene–T_4 conjugate.

Table 1. Values measured in ESI mode with an Esquire 3000 plus (or with an APPLEURA QSTAR pulsar I)[a] instrument

Compound	MW found	MW calcd.
T_4	1154	1154
Terthiophene–T_4	1508	1508
19-mer	5648.0[a]	5646.6
Terthiophene-19-mer	6002.4[a]	6001.0

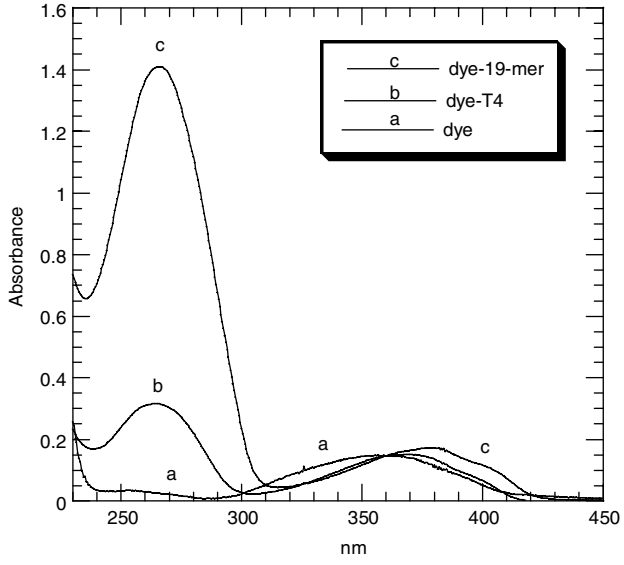

Figure 4. UV–vis spectra of compound **1** (a), terthiophene–T_4 (b), and terthiophene-19-mer (c) at a concentration of $7.6{\cdot}10^{-6}$ M in water.

260 nm and the dye maximum wavelength at 356 nm (Fig. 4).

Finally, the conjugates showed an intense bluish photoluminescence emission when excited at 354 nm (Fig. 5). The emission intensity allows an easy detection at

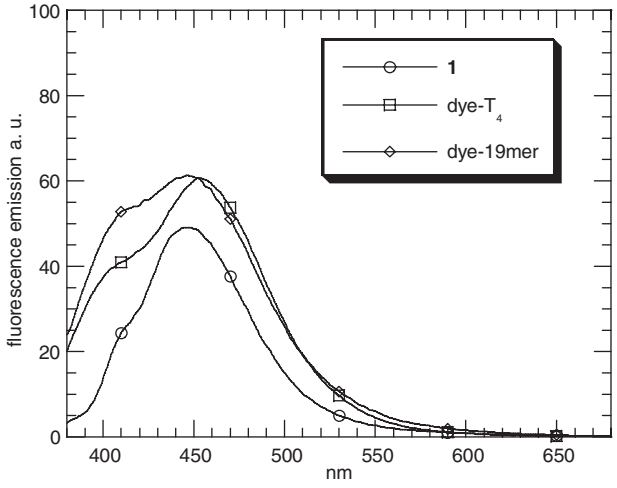

Figure 5. Photoluminescence spectra ($\lambda_{exc} = 354$ nm) of terthiophene **1** alone, and conjugated to T_4 and 19-mer. All compounds were 10^{-6} M in water.

sub-micromolar concentrations such as those usually required in the hybridization or microscopy studies. Moreover, the emission signal is stable under prolonged UV irradiation, contrary to other bioconjugates labelled with widely used fluorophores such as fluorescein whose emission decays in few minutes.

In conclusion, we have demonstrated, for the first time, the suitability of an oligothiophene, namely terthiophene **1**, for use as a fluorescent tag for oligonucleotides via phosphoroamidite coupling. This finding is important, because oligothiophenes are a large family of well-known compounds whose emission can easily be modulated by minor structural modifications to cover the full range of the visible spectrum. We infer that the electroactive properties of oligothiophenes could be exploited for the preparation of conjugates able to produce, during hybridization, an electrochemical trans-

duction signal that could lead to the realization of a biosensor device.[14,15] Our synthetic methodology should be applicable to oligothiophenes of different length and functionalization allowing the synthesis of a great variety of oligonucleotide conjugates with the desired characteristics. We are currently working to prepare such conjugates to study their fluorescence and redox properties, and their interaction with complementary strands.

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