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Oligothiophene Molecular Beacons

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Oligomers of thiophene are widely studied compounds for their electronic and optoelectronic properties. Despite their strong fluorescence, their use as markers for biomolecules, especially for oligonucleotides (ONs), is still largely unexplored. Here, we describe the synthesis of a series of ON molecular beacons employing different oligothiophenes as fluorescent probes and discuss their fluorescence emissions in comparative experiments with and without dabcyl as a quencher, in their hairpin and linear conformations, and as duplexes after hybridization with a complementary target.

INTRODUCTION

Oligomers of thiophene are well-studied compounds for their (opto)electronic properties. For instance, they have been used for the preparation of thin-film-based organic devices (LED, FET, etc.) (*1*, *2*). An important characteristic of this class of compounds is that their absorption and emission wavelength can be rationally predicted and easily obtained by a careful design of their molecular structure and positioning of substituents (*3*). We have recently started a program aimed at exploring the biological potential of this class of compounds as fluorescent markers for biomolecules such as proteins (*4*) and, more recently, oligonucleotides (*5*, *6*). The use of fluorescence as a tag for oligonucleotides is a useful alternative to radioactive labeling in quantitative experiments (*7*); it also allows an easy localization of oligonucleotides in cells (*8*). The emission of a fluorescent molecule is extremely sensitive to its environment, since small conformational changes of the fluorophore due to solvent effects or to interactions with other molecules through complex formation or energy transfer (FRET) may lead to dramatic changes in emitted light (*9*). In 1996, Tyagi showed a stunning application of FRET in the field of molecular recognition using double-labeled oligonucleotides called "molecular beacons" (*10*). Probes based on this principle are oligonucleotides (ONs) that spontaneously form a hairpin structure (see Figure 1) that puts their 5′ and 3′ ends in close proximity. In such a conformation, the fluorescence of a fluorophore tagged to one of the extremities can be quenched by a suitable moiety linked to the other end through Forster's energy transfer mechanism (*11*) and other mechanisms (*9*). When the ON probe in its quenched form is hybridized with a complementary strand, its conformation changes from hairpin to a distended double helix in which the fluorophore is moved away from the quencher and the fluorescence is (at least in part) restored. In ideal conditions, the ON probe behaves like a molecular beacon (MB): a strong fluorescence emission indicates the formation of the distended duplex (hence the presence of the target), while a weak response to the exciting light reveals the hairpin structure (indicating the absence of the target).

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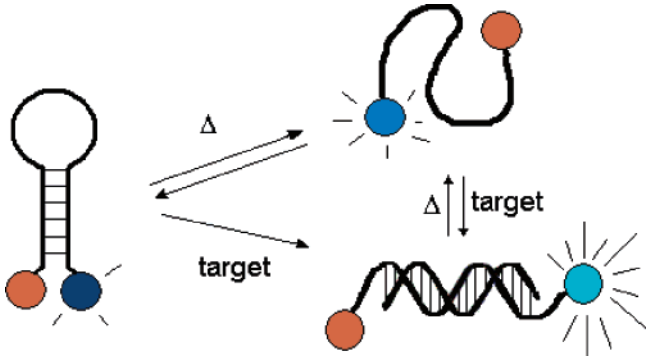

Figure 1. In the hairpin form, the fluorescence of the molecular beacon is quenched by the proximal quencher (orange circle). After denaturation by heat or after a hybridization event, the hairpin structure is broken and the quencher is moved away, restoring the fluorescence signal.

EXPERIMENTAL PROCEDURES

Synthesis and Purification of Oligonucleotides and Conjugates. The **MB** sequence was synthesized in our laboratory starting with commercial DMTr-C6-phtalimido-CPG. The compound was purified by ionic exchange chromatography on Sephacell using a gradient of triethylammonium hydrogen carbonate. Pure fractions were pooled, lyophilized several times, quantified, and split in vials of about 20 OD. The vials were eventually lyophilized and stored at −20 °C until use.

Similarly, the **dabcyl-MB** sequence was synthesized on a 10 μM scale on the same CPG, using DMTr-dabcyl-C6-amidite as the last monomer. This compound was purified on a C-18 reversed-phase column using a gradient of acetonitrile in 0.1 M of aq triethylammonium acetate. Pure fractions were collected, lyophylised several times, and split in amount of 20 OD in separate vials, stored at −20 °C.

The target sequence was purified by *n*-BuOH precipitation and found to be more than 80% pure at 260 nm by HPLC analysis on both reversed-phase and ionic exchange columns. It was kept solid at −20 °C until the moment of use.

Succinimidyl Esters. The succinimidyl esters of fluorophores **A**, **B**, and **D** (Figure 2) were synthesized according to the modalities described in ref 5. Succinimidyl esters of derivatives **C** and **E** are gifts from Mediteknology srl.

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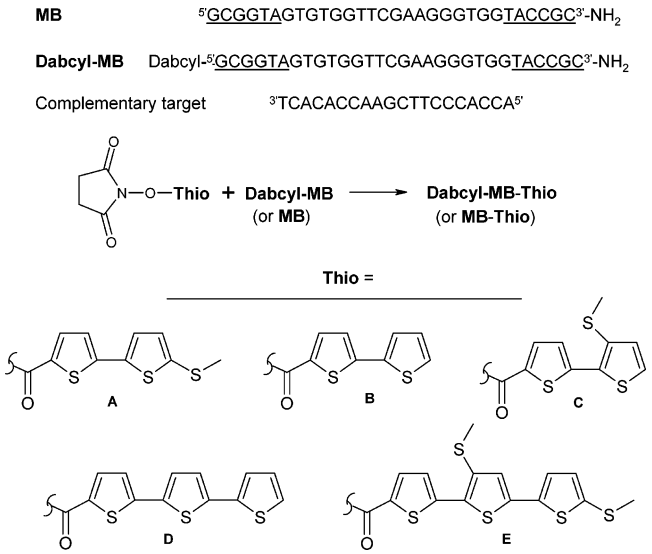

Figure 2. List of used compounds. The underlined bases form the hairpin stem.

Fluorescent ON Molecular Beacons. Fluorescent molecular beacons (with or without dabcyl) were obtained by mixing 20 OD of the corresponding 3′-amino-oligonucleotide with 1 mg of the succinimidyl ester of the oligothiophene in a buffer containing 160 μL of DMF and 18 μL of HEPES 0.2 M, pH 8.0, in water. The mixture was kept at 40 °C overnight. The reaction mixture was then diluted with 1 mL of water, and the excess of thiophene succinimidyl ester was extracted by repeated washing with dichloromethane. The aqueous phase was concentrated and purified by HPLC reversed-phase chromatography using a gradient of acetonitrile in 0.1 M aq TEAA. The collected fractions were judged more than 90% pure by absorbance at 260 nm and more than 95% pure by fluorescence detection using the appropriate λ_{ex} and λ_{em} for each compound. Usually, 5−7 OD of pure conjugate were obtained. The UV/vis profile of each conjugate was carefully checked to verify the correct incorporation of the oligothiophene and dabcyl. Finally, the identity of the ON MBs was confirmed by mass spectrometry (Bruker Esquire 3000 plus) in ESI mode with the following results: **MB-A** calcd *m*/*z* 9792.7, found 9791.4; **dabcyl-MB-A** calcd *m*/*z* 10255.1, found 10256.6; **MB-B** calcd *m*/*z* 9746.6, found 9746.3; **dabcyl-MB-B** calcd *m*/*z* 10209.0, found 10209.6; **MB-C** calcd *m*/*z* 9792.7, found 9792.7; **dabcyl-MB-C** calcd *m*/*z* 10255.1, found 10255.5; **MB-D** calcd *m*/*z* 9828.7, found 9831.2; **dabcyl-MB-D** calcd *m*/*z* 10291.1, found 10290.8; **MB-E** calcd *m*/*z* 9920.9, found 9920.0; **dabcyl-MB-E** calcd *m*/*z* 10383.3, found 10383.5.

Sample Preparation. To prevent manipulation errors, especially in the fluorescence readings, all experiments were done starting from a 1 μM concentration of the sample in a 3.0 mL fluorescence cuvette. The sample was then used for UV scan and fluorescence measurements, and then 5 μL of complementary target ON was added to the very sample to perform the hybridization experiments.

RESULTS AND DISCUSSION

In this work, we used fluorophores made of two and three thiophene rings (see Figure 2) tagged to the 3′-end of the ON strand, while the other end was kept free or conjugated to a dabcyl moiety.

The stem part of the sequence **MB** was chosen for the strong T_m found to be about 38 and 50 °C for **MB** and **dabcyl-MB** (data not shown), respectively, while its loop region was chosen to be complementary to an oligo already used by our group (*6*). The 3′-amino group was obtained by use of commercial DMTr-C6-phthalimido-CPG (Glen research). The use of dabcyl as a quencher was dictated by its large absorption region and by the commercial availability of its DMTr-dabcyl-C6-amidite (Chem Genes).

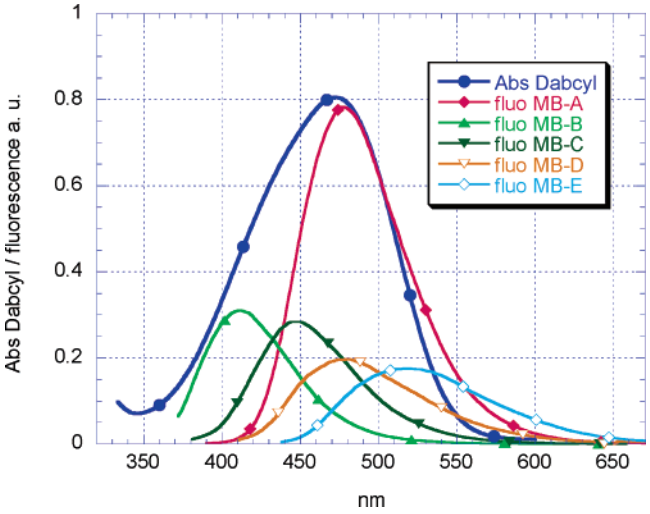

Figure 3. Superimposition of UV absorption spectrum of the dabcyl moiety of **dabcyl-MB** with fluorescence emissions of **MB-A**−**MB-E** conjugates. Each thiophene derivative was excited at its maximum excitation frequency. The intensities of the emission spectra, on an arbitrary scale, are proportional among all members. The absorption of the dabcyl derivative is also on an arbitrary scale. All fluorescence spectra were recorded at 1 μM concentration in 100 mM NaCl, 10 mM sodium cacodylate buffer pH 7.0.

Table 1. List of Maximum Frequency of Excitation and Emission of Succinimide and Oligonucleodide Derivatives

	succinimidyl ester		oligo conjugate	
entry	λ_{ex}	λ_{em}	λ_{ex}	λ_{em}
A	370	479	360	479
B	352	418	335	410
C	361	479	350	445
D	390	460	410	480
E	418	549	425	520

Oligothiophene fluorophores **A**−**D** were chosen for their intense fluorescence in water and for the overlap of their emission within the absorption range of the dabcyl as illustrated in Figure 3.

After conjugation, we noticed, in aqueous solution, some small variations at both wavelengths of absorption and emission of thiophene derivatives with respect to those of their succinimidyl ester parents in dichloromethane. A list of absorption and emission wavelength is reported in Table 1.

All the compounds were analyzed by the same methodology described below for **MB-A** and **dabcyl-MB-A**.

MB-A and **dabcyl-MB-A** were analyzed by UV at 10, 20, 30, 40, 50, 60, and 70 °C. The spectra had the expected UV/vis intensity ratio and did not shown any significant variation in the shape of the signal in the visible region, a strong indication that neither the tagged chromophore nor the quencher was involved in formation of complexes directly affected by temperature change (as would be expected in the case of intercalation of the dabcyl or thiophene moieties between base pairs of the stem of the hairpin of the molecular beacons) (see Figure 4). (A similar behavior was shown by all the other members, derivatives **B**−**E**, not shown.)

Compoud **MB-A** showed no clear melting point (from hairpin to random coil) in melting experiments, while the **dabcyl-MB-A**

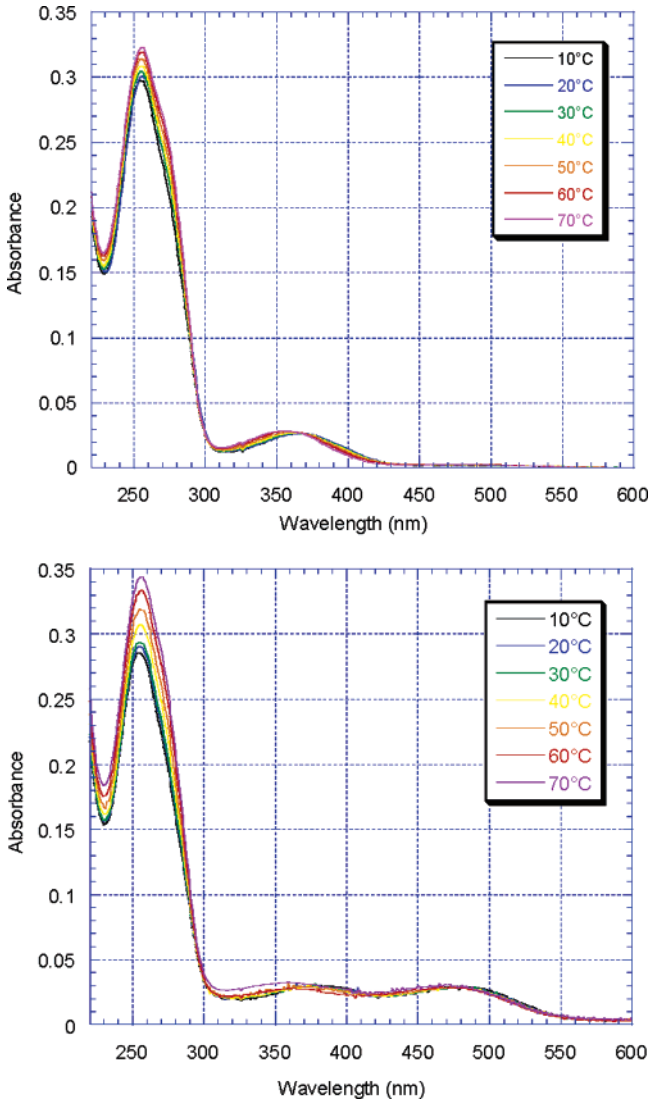

Figure 4. UV/vis scan at various *T* of **MB-A** (top panel) and **dabcyl-MB-A** (bottom panel) at 1 μM concentration in 100 mM NaCl and 10 mM sodium cacodilate pH 7.0.

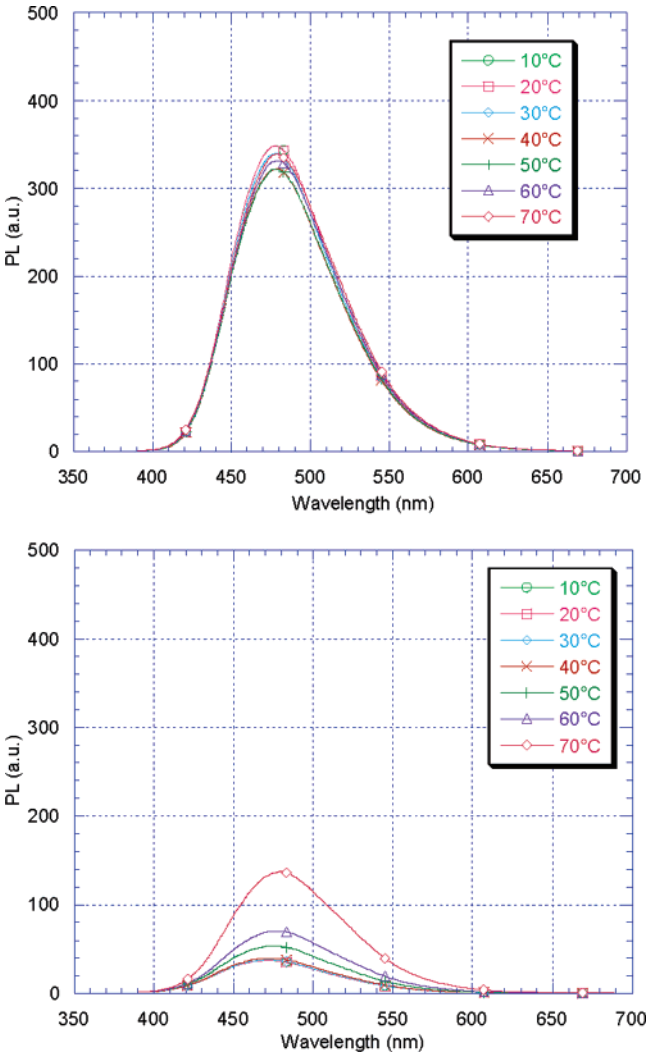

Figure 5. Variation of fluorescence with the temperature. Top panel **MB-A**; bottom panel **dabcyl-MB-A**.

showed a clear transition centered at 58 °C. (In all tested compounds, the melting point was not clearly detectable in the **MB** series, while in the **dabcyl** series, it was in the range 56−58 °C.)

Next, we analyzed the emitted fluorescence of **MB-A** and **dabcyl-MB-A** at different temperatures (Figure 5).

As expected, in the absence of dabcyl, the emission was slightly affected by heating; the intensity decreased by less than 10% from 10 to 70 °C (as in the corresponding succinimidyl parent compound; data not shown). This decrease can be attributed to a partial loss of coplanarity between the thiophene rings with heating. The **dabcyl-MB-A** showed a marked increase in fluorescence emission on going from 40 to 70 °C due to the thermal opening of the hairpin structure (molecular beacon effect). It is remarkable that even at high temperature the fluorescence emission of **dabcyl-MB-A** is lower than that of the corresponding compound without the quencher. This indicates that even in the random coil conformation the dabcyl moiety is able to quench part of the emission of the thiophene derivative **A** (dabcyl effect). This quenching was found to be independent of the concentration.

Next, we determined, by UV, the melting point of both **thiophene A** conjugates with their complementary strand (Figure 6).

Both molecular beacons showed a clear melting point (48 and 64 °C, respectively) higher than that of the hairpin, thus demonstrating that in the presence of the target the favored form is the duplex with the complementary strand.

Eventually, we studied the variation of fluorescence of the formed duplex with temperature (Figure 7).

By comparing all the above experiments, several conclusions can be drawn:

- The double labeling favors the stability of the hybrid with the target.
- The fluorescence of **MB-A** is enhanced (about 1.4 times) by hybridization (duplex effect).
- After hybridization with the target, the fluorescence of **dabcyl-MB-A** is more than 7 times higher than that of its hairpin form ("diagnostic effect": combination of molecular beacon and duplex effects).
- After hybridization, the fluorescence of **dabcyl-MB-A** is higher than that of the same compound in random coil form.

The experiments illustrated for **thiophene-A** derivatives were performed with all the compounds in Figure 2. A summary of the findings are shown in Figure 8.

Data in Figure 8 show that dabcyl is a quencher for all derivatives; however, while for derivatives **D** and **E** after hybridization with the complementary strand the fluorescence of the dabcyl derivative is equal to that of the relative compound without dabcyl, in cases **A** to **C**, the signal remains lower. This can indicate that for these derivatives the distance between the

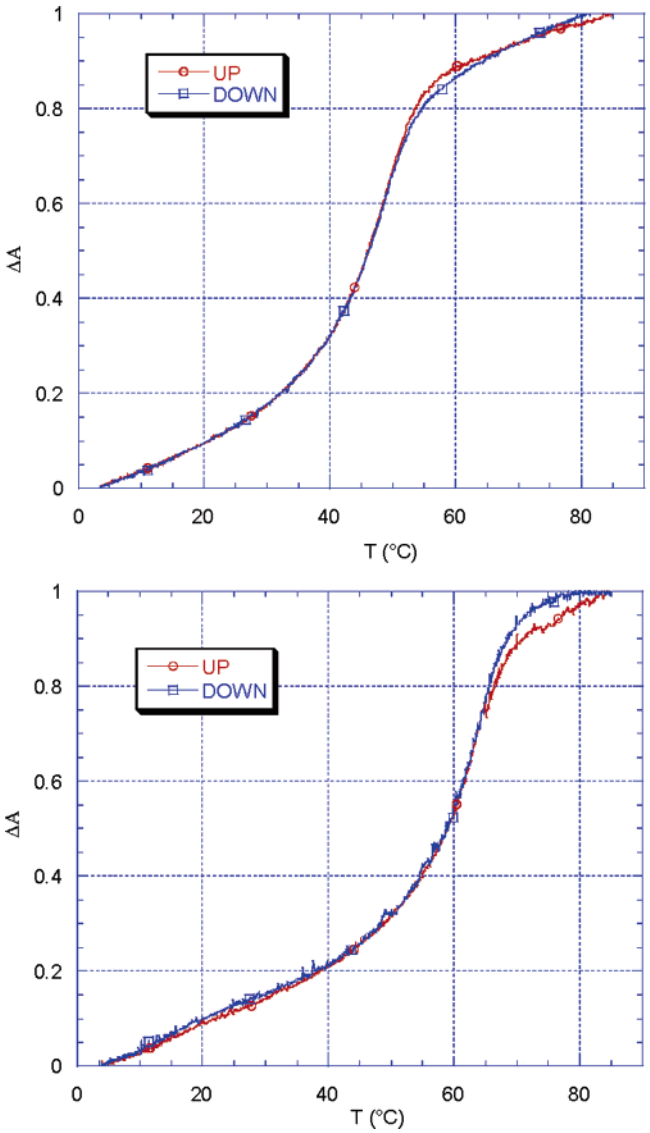

Figure 6. Melting of duplex **MB-A** (top panel) and **dabcyl-MB-A** (bottom panel) with their target (1.2 equiv). For each melting, heating and cooling curves are shown. The melting was performed on a buffer containing 100 mM NaCl and 10 mM sodium cacodilate at pH 7.0. Heating up and cooling down rates were of 0.2 °C/min. Normalized variations of absorbance were plotted.

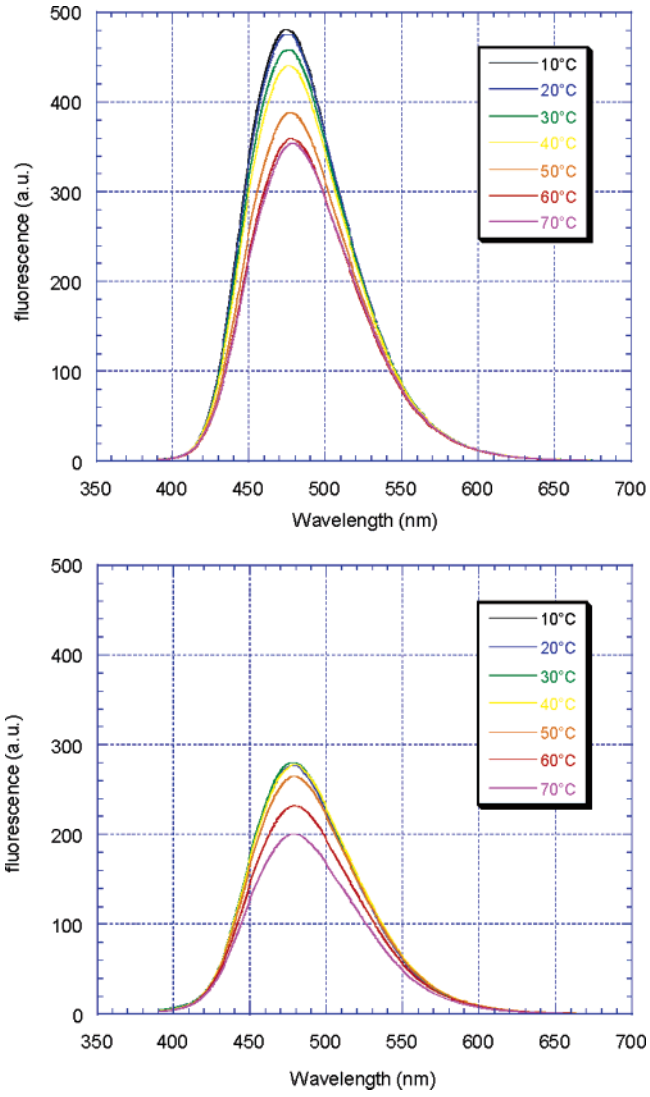

Figure 7. Variation of fluorescence emission with temperature for **MB-A** (top panel) and **dabcyl-MB-A** (bottom panel) after hybridization.

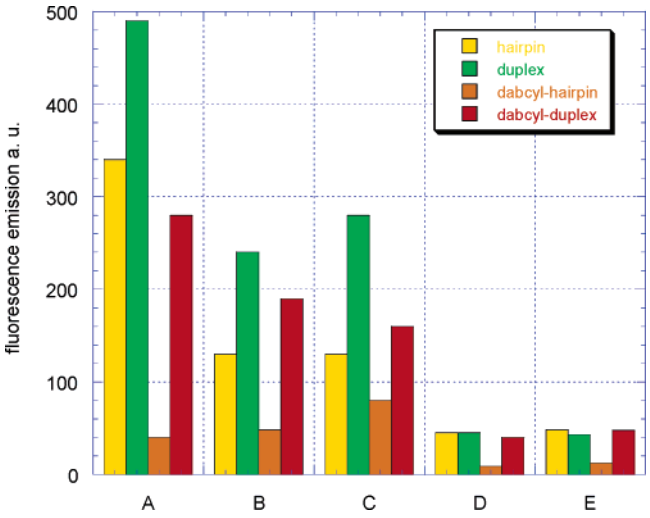

Figure 8. For each compound, the intensity of the emitted fluorescence at room temperature (on the same scale for all compounds) is reported. For each group, left to right: yellow bars, **MB hairpins**; green bars, **duplex MBs/target**; orange bars, **dabcyl-MB hairpins**; red bars, **duplex dabcyl-MBs/target**.

fluorophore and the quencher, even in the distended duplex, does not reach the Forster's radius. Oligonucleotide conjugates of **A**, **B**, and **C** are, at least within this system, quite sensitive to the hairpin−duplex transition even in absence of dabcyl as a quencher. We still do not know if this change of emission intensity depends on a possible quenching from bases on the 5′ region that came in proximity to the fluorophore in the hairpin structure (*12*, *13*) or if the formation of the duplex puts the fluorophore in a more favorable condition to increase the fluorescence quantum yield. Luckily for our systems, the molecular beacon effect and the duplex effect combine favorably to enhance the "diagnostic value" of probes **dabcyl-MB-A** and **dabcyl-MB-B**.

Derivatives **A**, **B**, and **C** (two thiophene rings) show a higher fluorescence than derivatives **D** and **E** (three thiophene rings), as well as their precursors; among the first three, derivative **A** has the strongest quantum yield.

In conclusion, we have described the fluorescence properties of a series of oligothiophene conjugated oligonucleotides. All compounds show a high fluorescence emission (they are easily detectable at 10−100 nM concentration). All derivatives are suitable for preparation of molecular beacons with dabcyl as a

quencher. Derivatives **A**−**C** are sensitive enough, at least in this system, to reveal a hybridization event even in absence of dabcyl.

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