Oligothiophene Isothiocyanates as a New Class of Fluorescent Markers for Biopolymers

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Abstract: The regioselective synthesis of fluorescent oligothiophene isothiocyanates is described. The isothiocyanates were reacted with bovine serum albumin (BSA) following standard procedures and the optical properties of the oligothiophene-BSA conjugates were analyzed as a function of oligomer concentration, time, and irradiation power. The oligothiophene-BSA conjugates were chemically very stable and their photoluminescence characteristics persisted unaltered for several months. Photoluminescence data relative to the conjugate of an oligothiophene-*S*,*S*-dioxide isothiocyanate with monoclonal anti-CD8 antibody are reported. No fluorescence quenching was observed following the binding of the isothiocyanate to the antibody and the conjugate displayed high chemical stability and photostability.

In the past few years optical probes and fluorescence detection have replaced radioisotopes as analytical tools in most biomedical applications. Fluorescent markers allow for the detection of the components of complex biological systems with great sensitivity and accuracy. There is an extensive literature on the use of fluorescent markers in biochemistry, pharmacology, and medicine. Fluorescence is the tool of choice to monitor antigen−antibody interactions in various bioanalytical applications, for use in immunochemistry, genomic studies, hystological assays, nucleic acids labeling, etc. Fluorescence detection is highly sensitive and noninvasive and enables one to monitor very fast processes, even on the order of picoseconds.[1−3]

A few families of fluorescent markers whith light-emitting frequencies spanning the entire visible spectrum and near-IR have been developed and are commercially available. Some of these are high-weight compounds, others are low-weight molecules with greater membrane permeability, and others are mixtures of compounds emitting light through a complicated pattern of intermolecular interactions.[1]

The most common fluorophores are amine-reactive compounds bearing functional groups capable of forming a stable linkage with biomolecules. One of the most used functional groups is the isothiocyanate group −N=C=S, which affords thioureas upon reaction with amines:

$$X-NCS + Y-NH_2 \rightarrow X-NH-C(S)-NH-Y$$

X = fluorescent marker, Y = biological molecule

The amino groups which are available for this reaction in proteins are the ϵ-amino groups of lisine residues. Bioconjugates of this type may be stable in many solvents and can even be stored for relatively long times.[1,2] Since the light emitted upon irradiation is proportional to the amount of fluorescent marker present in the bioconjugate, quantitative determinations can be made. Concentrations as low as picomolar and even femtomolar have been monitored.[1−3] Moreover, via chemical synthesis, the $-NH_2$ functionality can easily be grafted to the molecular skeleton of many biologically important compounds, such as, for example, oligodeoxynucleotides at the 5′ end position. In this way fluorophores containing the isothiocyanate functionality become useful for monitoring a variety of different processes of biological importance.[1]

Further developments of optical probe technology require the design of new fluorophores with better spectral properties, greater tunability of the emission frequency, ease of synthesis, and standardization of the procedures for binding biomolecules. Improvements in these fields would, in particular, extend the feasibility of multilabeling experiments, i.e., experiments allowing the simultaneous monitoring of different biochemical reactions.

An ideal class of fluorophores should have high absorbance values, large differences from absorption and photoluminescence frequencies (Stokes shifts), and high fluorescence quantum yields (the ratio of the number of photons emitted to the number of photons absorbed). Among fluorophores of current practical importance such as fluorescein and fluorescein derivatives[4] the

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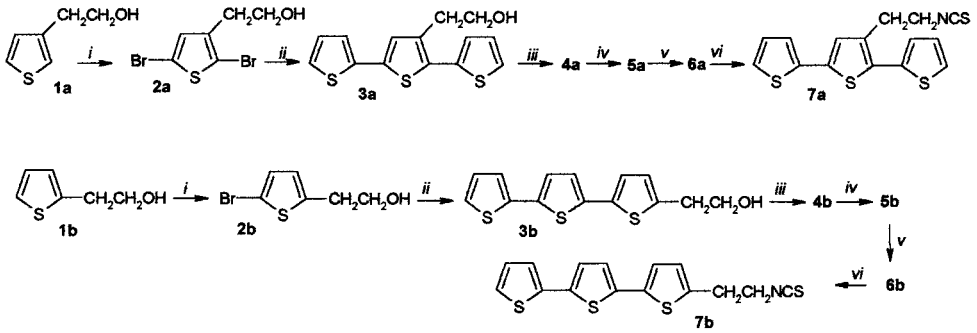
Scheme 1. Synthetic Pattern for the Synthesis of Thiophene Isothiocyanates Starting from Commercial 2-(3-Thienyl)ethanol **1a** and 2-(2-Thienyl)ethanol **1b**[a]



[a] Conditions: (i) NBS, toluene, −20 °C; (ii) 2-tributylstannylthiophene, $Pd(AsPh_3)_4$, toluene, 110 °C; (iii) CH_3SO_2Cl, CH_2Cl_2, Et_3N, −20 °C; (iv) NaN_3, DMF, 60 °C; (v) $LiAlH_4$, Et_2O; (vi) 2-pyridyl thiocarbonate, CH_2Cl_2.

molar extinction coefficients are in the range 5000−200000 cm^{-1} M^{-1} while the photoluminescence quantum yields vary from 0.05 to 1.0.[1,2]

High absorbance values and large Stokes shifts are characteristic of thiophene oligomers, a well-known class of compounds which have been actively investigated in recent years for their photo- and electroluminescence properties.[5] Time-resolved experiments have shown that the their photoluminescence lifetimes are on the order of nanoseconds[6a−c] and it has been demonstrated that they can reach high PL quantum yields in solution as well as in the solid state, depending on oligomer size and substitution pattern.[6a−e] Moreover, the solid-state PL frequencies of these compounds can be tuned across the visible range and near-IR with an astonishing number of shades by changing the oligomer size and the number and the position of various substitutents.[6e] Major advantages of thiophene oligomers are the chemical stability and the easy functionalization.[5] They are soluble in most organic solvents and with appropriate functionalization even in water.[7]

On these grounds thiophene oligomers appear suitable to investigate as fluorescent probes for biomolecules, but to the best of our knowledge no attempts in this direction have been made so far. Furthermore, despite the great number of functionalization types described in the literature for these compounds,[5] the functionalization with the isothiocyanate group has not yet been reported.

In this paper we describe the regioselective synthesis of fluorescent oligothiophene isothiocyanates and their conjugation with bovine serum albumin (BSA), the cheapest commercially available protein. The optical properties of oligothiophene-BSA conjugates were analyzed as a function of oligomer concentration, irradiation power, and resistance to prolonged irradiation. The paper also describes the optical properties of a conjugate of the anti-CD8 monoclonal antibody with an oligothiophene-*S*,*S*-dioxide isothiocyanate having the maximum wavelength absorption near 480 nm.

We will show that our results open the door to the investigation of oligothiophene isothiocyanates as a novel and promising class of fluorophores for biomolecular applications.[8]

Results

(I) Synthesis and Optical Properties of Oligothiophene Isothiocyanates. The synthetic pattern for the preparation of oligothiophene isothiocyanates is shown in Schemes 1 and 2, starting from commercial thiophene derivatives. Scheme 1 shows the synthesis of isothiocyanates **7a** and **7b** starting from 2-(3-thienyl)ethanol (**1a**) and 2-(2-thienyl)ethanol (**1b**).

Monomers **1a** and **1b** can easily be transformed into the corresponding isothiocyanates by the following pattern: alcohol → tosylate → azide → amine → isothiocyanate through steps iii to vi.[9−11] Moreover, these isothiocyanates are easily mono- or dibrominated at the terminal positions. Unfortunately, we were not able to find the experimental conditions to couple these bromo derivatives with thienyl stannanes or boronic acids and build up oligomers by means of the Stille[12] or Suzuki[13] reactions. Thus, we first incorporated the (thienyl)ethanol moiety into an oligothiophene and then transformed the OH functionality into the isothiocyanate group according to the modalities described in Scheme 1.

Tuning the absorption and emission wavelengths of thiophene oligomers requires changes in oligomer size and functionalization pattern. Thus, we found it more expedient to develop an alternative synthetic method in which the tranformation of a functional group into the isothiocyanate is carried out in the last step of the reaction and is almost quantitative. Scheme 2 illustrates the synthesis of isothiocyanates **10b**, **12c**, and **13b** starting from commercial thiophene and 2,2′-bithiophene.

The method illustrated in Scheme 2 takes advantage of the different reactivity of the chlorine atoms in chloro(chloromethyl)dimethylsilane ($ClCH_2Si(CH_3)_2Cl$), which is also a commercial product. The chlorine atom attached to silicon being much more reactive than the one attached to carbon, the $-CH_2-Si(CH_3)_2Cl$ moiety can be directly linked to the thiophene ring by lithiation followed by reaction with chloro(chloromethyl)-

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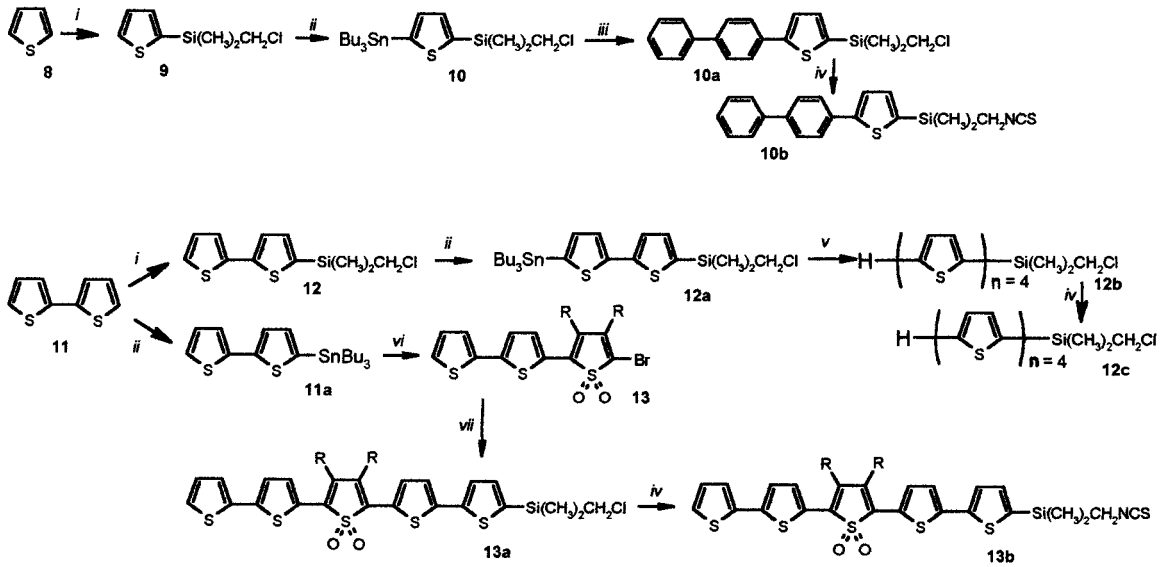
Scheme 2. Synthetic Pattern for the Preparation of Oligothiophene Isothiocyanates by Means of Chloro(chloromethyl)dimethylsilane[a]



[a] Conditions: (i) LDA,$ClCH_2Me_2SiCl$; (ii) BuLi, Bu_3SnCl; (iii) 4-biphenylbromide, $Pd(AsPh_3)_4$, toluene, 110 °C; (iv) NaSCN, acetone, Et_2O; (v) 5-bromo-2,2′-bithiophene, $Pd(AsPh_3)_4$, toluene, 110 °C; (vi) 2,5-dibromo-3,4-dihexyl-thiophene-1,1-dioxide,[14a] $Pd(AsPh_3)_4$, toluene, 110 °C; (vii) 5-dimethyl(chloromethyl)silyl]- 5′-tributylstannyl-2,2′-bithiophene, $Pd(AsPh_3)_4$, toluene, 110 °C.

Table 1. Maximum Absorption (λ_{max})[a] and Emission (λ_{PL})[a,b] Wavelengths and Molar Extinction Coefficients (ϵ)[c] of Isothiocyanates **7a**, **7b**, **10b**, **12c**, and **13b**

compd	λ_{max}	ϵ	λ_{PL}	$\Delta(\lambda_{PL} - \lambda_{max})$[d]
7a	342	11 800	434	6199
7b	360	18 950	438	4947
10b	312	42 000	450	9829
12c	396	47 000	560	7395
13b	477	38 000	650	5579

[a] In nm, in methylene chloride. [b] $\lambda_{exc} = 325$ nm. [c] Units: $cm^{-1}\ M^{-1}$. [d] Stokes shifts are given in cm^{-1}.

dimethylsilane. Afterward the chlorine attached to carbon may be transformed quantitatively into the corresponding isothiocyanate by reaction with sodium thiocyanate NaSCN.

The optical properties of isothiocyanates **7a**, **7b** and **10b**, **12c**, **13b** are shown in Table 1. It is seen that the wavelengths of maximum absorption span from 312 to 477 nm with extinction molar coefficients varying from 11 800 to 47 000 $M^{-1}\ cm^{-1}$.

The light emission wavelengths of **7a,7b** and **10b**, **12c** and **13b** cover the entire visible spectrum from 434 to 650 nm, i.e., from blue to red. The Stokes shifts from absorption to emission wavelengths are large and vary from 78 nm (0.61 eV) for trimer **7b** to 173 nm (0.69 eV) for pentamer **13b**.

Figure 1A shows the absorption spectra of compounds **10b**, **12c**, and **13b** and Figure 1B the photoluminescence spectra of the same compounds upon irradiation with the same laser source (λ_{exc} = 325 nm). As generally observed for thiophene oligomers[6a−b,d] the absorption spectra of **10b**, **12c**, and **13b** are shapeless and broad. This makes it possible to oberve significant photoluminescence intensities by exciting with the same light source compounds having different maximum absorption wavelength (cf. also ref 6e). The photoluminescence spectra of **10b** and **12c** display fine structure, as is generally observed for conventional oligothiophenes,[6a,b] while the photoluminescence spectrum of **13b** displays a shapless band, typical of oligothiophene-*S*,*S*-dioxides.[6e−g]

Within the limits of the experimental error the PL quantum yields (QYs) of oligothiophene isothiocyanates in chloroform were the same as those of the unsubstituted parent compounds,

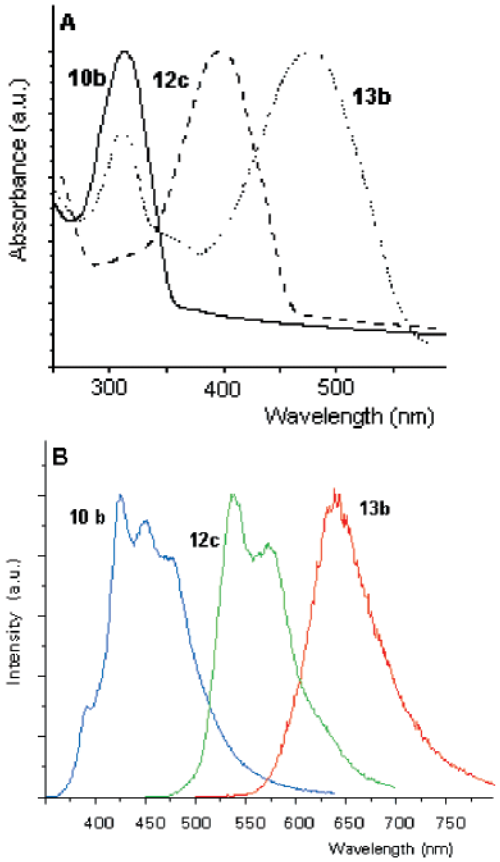


Figure 1. (A) Absorption and (B) photoluminescence spectra of isothiocyanates **10b**, **12c**, and **13b** in methylene chloride (λ_{exc} = 325 nm).

indicating that the presence of the NCS groups scarcely affects this parameter. For compounds **7a** and **7b** we measured values in the range 0.08−0.1, for **12c** a value in the range 0.15−0.2, for **10b** a value between 0.4 and 0.5, and for **13b** a value of 0.01. These QYs are similar to those reported in the literature for unsubstituted ter- and quaterthiophene in solution,[6a,b,d] for

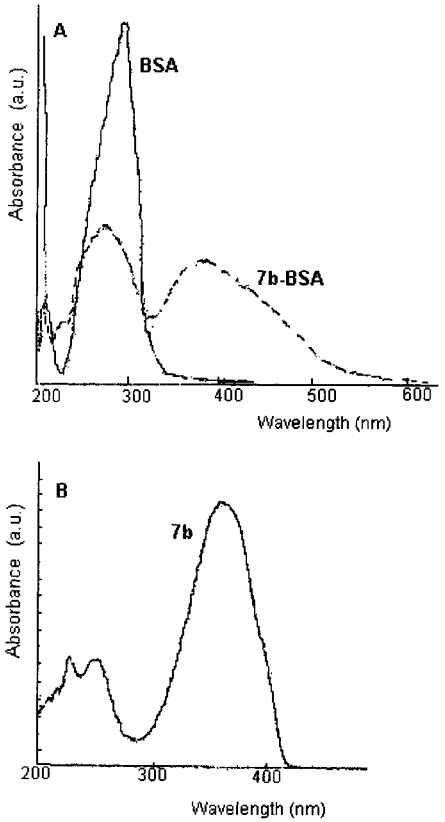


Figure 2. (A) Absorption spectrum of native bovine serum albumin (BSA) and of the conjugate **7b**-BSA. (B) Absorption spectrum of isothiocyanate **7b** in DMSO.

p-terphenyl in solution,[15] and for conformationally mobile oligothiophene-*S*,*S*-dioxides.[16] Nevertheless, these values are scarcely significant to our purposes as it is known that the PL quantum yields of thiophene oligomers depend to a great extent on their conformational mobility and that the rigidification of the system may lead to a significant increase in photoluminescence intensity.[6c] In consequence, the effective response of these fluorophores to UV excitation will depend on the degree of conformational freedom left after conjugation with the biomolecules.

(II) Conjugation with Bovine Serum Albumin (BSA). To test the feasibility of the conjugation of thiophene isothiocyanates with biopolymers, purified bovine serum albumin (BSA, fraction V) was first used as the representative protein.

The conjugation of oligothiophene isothiocyanates with BSA was carried out at basic pH, according to standard modalities (see the Experimental Section). The oligothiophene-BSA conjugate was separated from unbound isothiocyanate by gel filtration chromatography in saline phosphate buffer solution (PBS, pH 7.2).

Figure 2A shows the absorption spectrum of native BSA and that of the purified conjugate of **7b** with BSA in phosphate buffer solution while Figure 2B shows the absorption spectrum of pure **7b** for comparison.

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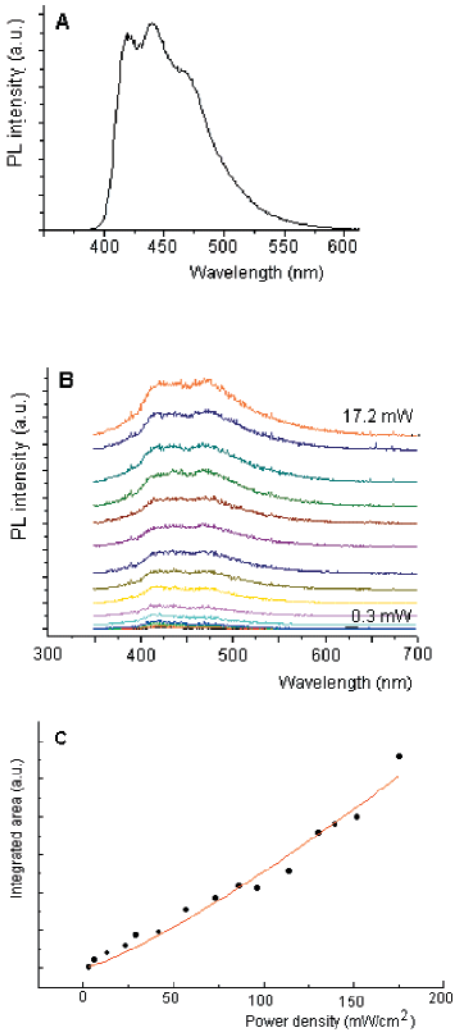

Figure 3. (A) Photoluminescence spectrum of isothiocyanate **7b** in DMSO. (B) Photoluminescence spectrum of conjugate **7b**-BSA. (C) Integrated area of the photoluminescence spectrum of **7b**-BSA vs excitation power density. $\Lambda_{exc} = 325$ nm.

The photoluminescence (PL) spectrum of the **7b**-BSA conjugate in PBS is shown in Figure 3B ($\lambda_{exc} = 325$ nm) for different values of the excitation power, while Figure 3A shows the PL spectrum of pure **7b** for comparison. Figure 3C shows the integrated area of the PL spectrum of the **7b**-BSA conjugate vs the excitation power density (mW cm^2). It is seen that the intensity of the PL spectrum increases almost linearly as a function of the power of excitation. No changes in the shape of the PL spectrum were observed.

As mentioned in the Introduction, the −NCS functionality reacts with the available ϵ-NH_2 groups of lysine residues of the protein. Thus, there is a threshold value for the number of isothiocyanate groups that can link BSA. The larger the number of fluorophores conjugated to the protein the greater the resulting photoluminescence intensity that can be obtained from the conjugate.

To establish the maximum PL intensity that can be obtained from a solution of **7b**-BSA for a given excitation power ($\lambda_{exc} = 325$ nm, excitation power = 10.5 mW) we first determined the reaction time needed to achieve the complete reaction between **7b** and BSA. Then, we determined the maximum number of **7b** molecules bound to BSA without protein denaturation.

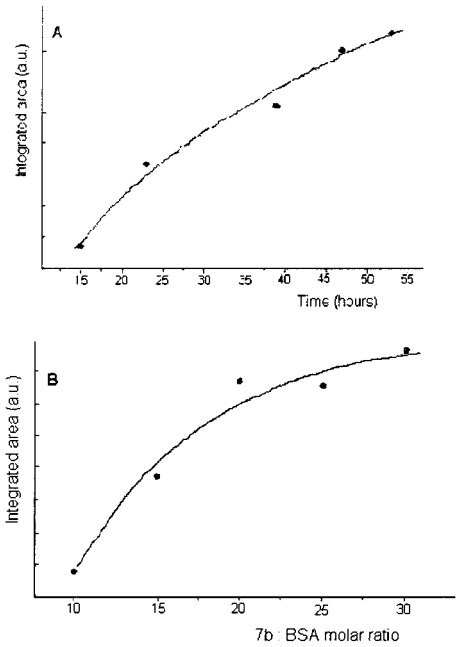

Figure 4. (A) Integrated area of the photoluminescence spectrum of the conjugate **7b**-BSA vs the reaction time at constant molar ratio (**7b**:BSA 10:1). (B) Integrated area of the photoluminescence spectrum of the conjugate **7b**-BSA vs **7b**:BSA molar ratio at constant reaction time (50 h).

To this aim five different samples containing **7b** and BSA in a 10:1 molar ratio were prepared, the reaction stopped at different times after mixing the reagents, and the conjugate separated from unbound components by gel filtration chromatography. Figure 4A shows the integrated area of the PL spectra of the five solutions vs reaction time. It is seen that the intensity of the PL signal increases as the reaction time increases up to nearly 50 h.

Afterward we prepared five different samples containing **7b** and BSA in 10:1, 15:1, 20:1, 25:1, and 30:1 molar ratios, the mixtures were allowed to react for about 50 h and then purified by gel filtration chromatography. Figure 4B shows the integrated area of the PL spectra of the **7b**-BSA conjugate as a function of the **7b**:BSA molar ratio. The figure shows that saturation occurs near 30 nmol of isothiocyanate per nmol of BSA. Up to this value the conjugate is soluble in saline buffer solution (pH 7.2). Increasing the molar ratio beyond 30:1 causes the precipitation of the conjugate. Since it is known that there are 59 lysine residues in the protein BSA, these data indicate that half of the lysine residues of BSA are available for conjugation with isothiocyanate **7b** without protein precipitation.

Finally, to have an estimate of the stability of the **7b**-BSA conjugate we measured the intensity of the PL spectrum vs the exposure to UV irradiation. The sample was excited at λ_{exc} = 325 nm (excitation power = 10.5 mW) and the maximum intensity of the PL spectrum was monitored vs time. Figure 5 shows that after 45 h of continuous exposure to laser stream the maximum intensity of the PL spectrum of the **7b**-BSA conjugate is still more than half the starting value.

Very similar results were obtained with the conjugates of isothiocyanates **10b, 12c**, and **13b** with BSA. These conjugates were prepared in the same experimental conditions used for the **7b**-BSA conjugate and their response to irradiation power and irradiation time was very similar to those described above. As an example, Figure 6 shows the variations in the PL spectrum intensities of the conjugate of **13b** with BSA vs the **13b**:BSA molar ratio. The figure shows that the signal intensity increases

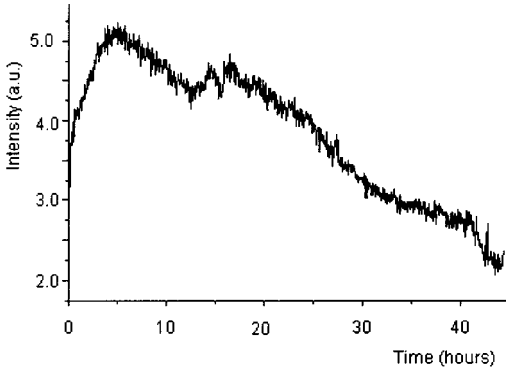

Figure 5. Maximum intensity of the photoluminescence spectrum of the conjugate **7b**-BSA vs time under continuous irradiation with λ_{exc} = 325 nm and irradiation power 10.5 mW.

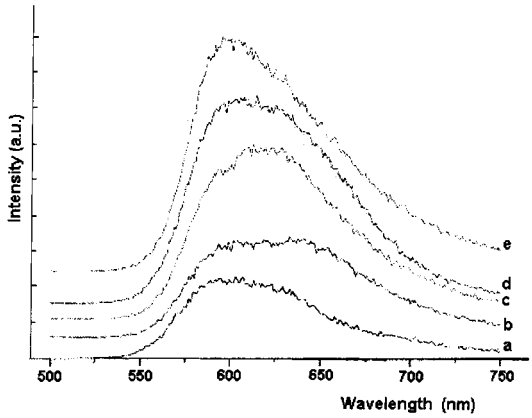

Figure 6. Photoluminescence spectra (λ_{exc} = 488 nm) of the conjugate of **13b** with BSA vs **13b**:BSA molar ratios: (a) 5:1, (b) 10:1, (c) 20:1, (d) 25:1, (e) 30:1.

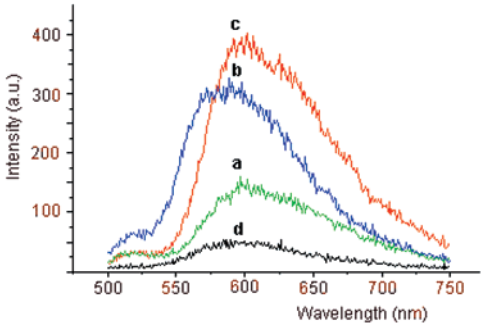

Figure 7. Photoluminescence spectra (λ_{exc} = 488 nm) of the conjugate of **13b** with antibody anti-CD8 at different **13b**:antibody molar ratios: (a) 5:1, (b) 10:1, (c) 25:1. Spectrum d is that of pure **13b** in phosphate buffer solution at a concentration comparable to that of spectrum a.

as the **13b**:BSA molar ratio increases with much the same trend as that observed for the conjugate of **7b** with BSA. One can remark that the shape of the PL spectrum of the conjugate of Figure 6 changes slightly on changing the components molar ratio. At the present state of knowledge it is difficult to ascribe this behavior to a specific phenomenon, although one can reasonably believe that it is related to the conformational mobility of the isothiocyanates. We limit ourselves to oberve that a similar behavior is also observed for the antibody-isothiocyanate (see below) reported in Figure 7 and that the phenomenon is worth analyzing in future, more detailed, investigations.

(III) Conjugation of Isothiocyanate 13b with anti-CD8 Monoclonal Antibody. The rationale behind the use of isothio-

cyanate **13b** is that currently available commercial instruments for flow cytometry applications employ the 488 nm spectral line of the argon-ion laser as the excitation source. This requires use of a fluorescent probe characterized by an absorption maximum wavelength near 480 nm. As shown in Figure 1, this is the case of isothiocyanate **13b** whose maximum absorption wavelength is $\lambda_{max} = 477$ nm.

Anti-CD8 is a IgG1 isotype-specific antibody which reacts with a 30/32 kDa molecular weight antigen normally expressed on the surface of the subpopulation of T-lymphocyte (suppressor cells) having cytotoxic activity.[17] The anti-CD8 is commonly used for diagnostic purposes in flow cytometry and immunohistochemical applications. Conjugates of anti-CD8 with fluorescein isothiocyanate (isomer 1, FITC) are commercially available and widely used.

Isothiocyanate **13b** dissolved in anhydrous dimethyl sulfoxide was reacted with anti-CD8 antibody in carbonate buffer solution (see the Experimental Section) in 5:1 (a), 10:1 (b), and 25:1 (c) **13b**/anti-CD8 molar ratios. The conjugation reaction was carried out for a time equivalent to that required to achieve the maximum conjugation of **7b** with BSA. At this stage no attempts to optimize the reaction time of **13b** with the antibody were made. After about 50 h at ambient temperature the unreacted **13b** was separated by the **13b**/anti-CD8 conjugate through a desalting column.

Figure 7 shows the photoluminescence spectra ($\lambda_{exc} = 488$ nm) of the conjugate of **13b** with antibody anti-CD8 for different **13b**:antibody molar ratios: 5:1 (a), 10:1 (b), and 25:1 (c). For comparison, also the spectrum of pure **13b** in phosphate buffer solution is reported (d) at a concentration comparable to that of spectrum a.

Comparison of spectra a, b, and c shows that the emission intensity increases as the **13b**:anti-CD8 molar ratio increases, in the same way as for the **13b**:BSA conjugate.

The three **13b**/anti-CD8 conjugate solutions corresponding to spectra a, b, and c were checked by flow cytometry to test the activity of the antibody in detecting the specific antigen present on the T-limphocyte cell surface. It was found that in the conjugate the activity of the antibody was completely preserved. The solutions were tested systematically for a period of three months. No changes in their properties and no traces of precipitate were observed.

Finally, it is worth noting that Figure 7 shows also that the intensity of the PL spectrum of pure **13b** is lower than that of the **13b**/anti-CD8 conjugates, indicating that the conjugation with the antibody does not lead to photoluminescence quenching of the fluorophore.

Discussion

The interpretation of the data reported in this paper is straigthforward. We have indeed demonstrated that it is possible to selectively derivatize thiophene oligomers with the isothiocyanate group and that oligothiophene isothiocyanates are photoluminescent compounds whose PL frequencies can be tuned across the visible range from blue to red. We have also demonstrated that they can be linked to proteins and give rise to photoluminescent fluorophore−protein conjugates with excellent chemical stability and photostability, which can undergo repeated excitation/emission cycles without fluorescence quenching.

Conjugation of the isothiocyanates with proteins—based on either conventional thiophene oligomers or oligothiophene-*S*,*S*-dioxides—was easily carried out employing standard methods and the conjugates were soluble and stable in phosphate buffer solution (pH 7.2) even for months.

As shown in Figures 4B and 6 for the conjugates of isothiocyanates **7b** and **13b** with bovine serum albumin and in Figure 7 for the conjugate of **13b** with the anti-CD8 antibody conjugate, the PL intensity increases progressively as the fluorophore−protein molar ratio increases. Fluorophore:protein molar ratios as high as 25/30:1 can be achieved without precipitation of the conjugate.

There is no irreversible destruction of the fluorophore under high-intensity excitation conditions (Figure 3B) or continuous irradiation for many hours. An extreme experiment such as that reported in Figure 5 for the **7b**-BSA conjugate shows that after more than 1 day under continuous laser irradiation the light-emitting properties of the fluorophore have not yet been substantially modified.

Our data show that there is no quenching of fluorescence of oligothiophene isothiocyanates following conjugation with the proteins. This is particularly relevant in the case of the **13b**/anti-CD8 antibody conjugate as antibodies often act as fluorescence quenchers owing to several different effects, including charge-transfer interactions with aromatic amino acid residues. Figure 7 shows that the conjugation to the antibody even increases the emission properties of **13b**, probably due to the increase in the intrinsic PL quantum yield of the fluorophore related to a partial loss of conformational freedom upon conjugation.

We believe that it is the presence of the $-Si(Me_2)CH_2-$ or $-CH_2CH_2-$ spacers that prevents the occurrence of strong intermolecular interactions between oligothiophene isothiocyanate and protein components and then prevents fluorescence quenching. Moreover, the flexibility of the synthetic methodology described here also allows, if needed, for spacer elongation by a few more methylene components and then for studies of fluorescence intensity dependence on chain elongation.

Contrary to the case of some families of fluorophores that exist as two or more isomers,[1] oligothiophene isothiocyanates may be prepared in highly pure form by regioselective synthesis.

They emit light across the entire visible range (see Table 1 and Figure 1) and their light-emission frequencies are expected to be tunable with an unprecedented variety of shades in the visible range and even in the near-IR. Indeed, the insertion of the isothiocyanate group at one terminal position or at one of the inner rings only causes minor alterations in the degree of π electron conjugation of the aromatic backbone. Thus, the absorption and emission properties are expected to be tunable in much the same way as already demonstrated for oligothiophene-*S*,*S*-dioxide.[6e]

The bandwidths of the absorption spectra of the isothiocyanates described here are large but the Stokes shifts between absorption and emission frequencies are also very large, as shown in Table 1. Thus, multilabel applications requiring identification of several different molecules by their fluorescence should be possible.

Being uncharged, oligothiophene isothiocyanates are pH-insensitive probes. Being low-weight molecules they should also

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be more membrane-permeant than many currently used fluorophores and be more useful for analyses with living cells.

We are aware of the fact there is a great deal of research still to be carried out before assuming that oligothiophene isothiocyanates are a convenient alternative to many currently used fluorophores: from engineering oligothiophene isothiocyanates with optimized absorbances and fluorescence quantum yields to optimizing reaction times and conditions for the preparation of the conjugates and defining a conjugation protocol applicable for all colors and from measuring the fluorescence quantum yields of the conjugates relative to those of the free fluorophores to functionalizing the fluorophores with groups capable of ensuring good solubility in water to demonstrating the feasibility of multilabeling experiments and so on. Nevertheless, we believe it is safe to draw the conclusions that follow.

Conclusions

Oligothiophene isothiocyanates are worth studying as fluorescent probes for detecting and analyzing biopolymers.

These compounds have indeed the requisite combination of being easy to conjugate to biomolecules with standard procedures, giving rise to chemically and optically stable conjugates, having fluorescence frequencies covering the entire visible spectrum, having a large extinction coefficient for absorption and large Stokes shifts from absorption to emission, and being amenable to the engineering of several different properties such as the distance of the fluorophore from the biopolymer or the solubility in water.

Finally, it is worth noting that besides the functionalization with the isothiocyanate group described in this paper the great versatility of thiophene chemistry will allow for the functionalization of thiophene oligomers with many other functional groups capable of binding proteins or DNA components, oligodeoxynucleotides, vitamins, hormones, etc, ..., greatly increasing in this way the range of applicability of the new class of fluorophores.

Experimental Section

Synthesis of Oligothiophene Isothiocyanates. 2-(2-Thienyl)ethanol (**1a**), 2-(3-thienyl)ethanol (**1b**), thiophene (**8**), 2,2′-bithiophene (**11**), 2-(tributylstannyl)thiophene, di-2-bipyridil thiocarbonate, 4-biphenyl bromide, and butyllithium were purchased from Aldrich.

The syntheses of 2,5-dibromo-3-(2-hydroxyethyl)thiophene (**2a**), 2-bromo-5-(2-hydroxyethyl)thiophene (**2b**), 3′-(2-methanesulfonyloxyethyl)-2,2′:5′,2″-terthiophene (**4a**), 5-(2- methanesulfonyloxyethyl)-2,2′:5′,2″-terthiophene **(4b)**, 3′-(2-azidoethyl)-2,2′:5′,2″-terthiophene (**5a**), 5-(2-azidoethyl)-2,2′:5′,2″-terthiophene (**5b**), 3′-(2-aminoethyl)-2,2′:5′,2″-terthiophene (**6a**), 5-(2-aminoethyl)-2,2′:5′,2″-terthiophene (**6b**), 2-[dimethyl(chloromethyl)silyl]-5-tributylstannylthiophene (**10**), 5-[dimethyl(chloromethyl)silyl]-2,2′-bithiophene (**12**), 5-[dimethyl(chloromethyl)silyl]-5′-tributylstannyl-2,2′-bithiophene (**12a**), and 2-bromo-3,4-dihexyl-5-[5-(2,2′-bithienyl)]thiophene-1,1-dioxide (**13**) are reported as Supporting Information.

3′-(2-Hydroxyethyl)-2,2′:5′,2″-terthiophene (3a). To a 40 mL toluene solution containing 0.25 mmol of $(Ph_3As)_4Pd$ prepared in situ was added 1.43 g (5 mmol) of **2a** and 3.73 g (10 mmol) of 2-(tributylstannyl)thiophene. The mixture was refluxed for 16 h. After evaporation of toluene the residue was chromatographed on silica gel with petroleum ether/ethyl acetate 7:3 as eluent. Product **3a** was obtained as a greenish solid (1.02 g, yield 70%): mp 54−55 °C, MS *m*/*e* 292 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 344 nm; IR ν_{OH} (CCl_4) 3634 cm^{-1}; ^1H NMR ($CDCl_3$, TMS/ppm) δ 7.31 (dd, ^3J = 5.2 Hz, ^4J = 1.2 Hz, 1H), 7.22 (dd, ^3J = 5.2 Hz, ^4J = 1.2 Hz, 1H), 7.17 (m, 2H), 7.07 (s, 1H), 7.07 (dd, ^3J = 5.2 Hz, ^4J = 3.2 Hz, 1H), 7.01 (dd, ^3J = 5.2 Hz, ^4J = 3.2 Hz, 1H), 3.90 (t, 2H), 3.02 (t, 2H), 1.72 (s, 1H, OH); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 136.8, 135.7, 135.7, 135.2, 131.1, 127.8, 127.5, 126.4, 126.3, 125.7, 124.6, 123.7, 62.6, 32.4.

5-(2-Hydroxyethyl)-2,2′:5′,2″-terthiophene (3b). This compound was obtained with the same procedure described for **3a**, using monobromo **2b** and 2-tributylstannyl-2,2′-bithiophene.[14] Yellow brown polycrystalline solid: mp 97−98 °C; MS *m*/*e* 292 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 360 nm; IR ν_{OH} (CCl_4) 3635, 3600 cm^{-1}; ^1H NMR ($CDCl_3$, TMS/ppm) δ 7.21 (dd, ^3J = 5.2 Hz, ^4J = 1.2 Hz, 1H), 7.16 (dd, ^3J = 5.2 Hz, ^4J = 1.2 Hz, 1H), 7.06 (d, ^3J = 3.6 Hz, 1H), 7.02 (m, 3H), 6.81 (dt, ^3J = 3.6 Hz, ^4J = 0.8 Hz, 1H), 3.88 (t, 2H), 3.05 (t, 2H), 1.67 (s, 1H, OH); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 140.3, 137.1, 136.3, 135.8, 135.7, 127.8, 126.5, 124.4, 124.2, 123.8, 123.6, 123.5, 63.3, 33.6.

3′-(2-Isothiocyanatoethyl)-2,2′:5′,2″-terthiophene (7a). To a solution of 70 mg (0.30 mmol) of di-2-pyridyl thiocarbonate (DPT) in 1 mL of CH_2Cl_2 were added 87 mg (0.30 mmol) of amine **6a** in 2 mL of CH_2Cl_2. The reaction mixture was stirred for 3 h at room temperature and then washed three times with water. The organic layer was dried and evaporated and the residue was chromatographed on silica gel with petroleum ether/ethyl acetate 9:1 as eluent. The product was isolated as a yellow brown oil (77 mg, yield 77%): MS *m*/*e* 333 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 342 nm; IR ν_{NCS} (CCl_4) 2083 cm^{-1}; ^1H NMR ($CDCl_3$, TMS/ppm) δ 7.36 (dd, ^3J = 5.2 Hz, ^4J = 1.2 Hz, 1H), 7.24 (dd, ^3J = 5.2 Hz, ^4J = 1.2 Hz, 1H), 7.19 (dd, ^3J = 3.6 Hz, ^4J = 1.2 Hz, 1H), 7.14 (dd, ^3J = 3.6 Hz, ^4J = 1.2 Hz, 1H), 7.09 (dd, ^3J = 5.2 Hz, ^4J = 3.6 Hz, 1H), 7.05 (s, 1H), 7.03 (dd, ^3J = 5.2 Hz, ^4J = 3.6 Hz, 1H), 3.74 (t, 2H), 3.14 (t, 2H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 136.5, 136.4, 134.5, 134.1, 131.7, 131.4 (NCS), 127.9, 127.7, 126.7, 126.1, 125.8, 124.8, 124.0, 45.2, 29.7. Anal. Calcd for $C_{15}H_{11}NS_4$: C 54.02; H 3.32. Found: C 54.18; H 3.33.

5-(2-Isothiocyanatoethyl)-2,2′:5′,2″-terthiophene (7b). Following the same procedure as above, a light yellow solid was obtained: mp 97−98 °C; MS *m*/*e* 333 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 360 nm; IR ν_{NCS} (CCl_4) 2080 cm^{-1}; ^1H NMR ($CDCl_3$, TMS/ppm) δ 7.22 (dd, ^3J = 5.2 Hz, ^4J = 0.8 Hz, 1H), 7.17 (dd, ^3J = 3.6 Hz, ^4J = 1.2 Hz, 1H), 7.07 (d, ^3J = 3.6 Hz, 1H), 7.02 (m, 3H), 6.81 (dt, ^3J = 3.6 Hz, ^4J = 0.8 Hz, 1H), 3.76 (t, 2H), 3.17 (t, 2H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 138.1, 137.0, 136.4, 136.1, 135.9, 131.8 (NCS), 127.9, 127.2, 124.5, 124.2, 124.1, 123.7, 123.6, 46.3, 30.9. Anal. Calcd for $C_{15}H_{11}NS_4$: C 54.02; H 3.32. Found: C 54.12; H 3.31.

2-(4-Biphenyl)-5-[dimethyl(chloromethyl)silyl]thiophene (10a). To a 10 mL toluene solution containing 0.015 mmol of $Pd(AsPh_3)_4$ prepared in situ was added 0.116 g (0.5 mmol) of 4-biphenyl bromide and 0.24 g (0.5 mmol) of compound **10**. The mixture was refluxed for 8 h. After evaporation of toluene the residue was chromatographed on silica gel with petroleum ether as eluent to give 0.11 g (yield 64%) as a white solid: mp 132−133 °C; MS *m*/*e* 342 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 314 nm; ^1H NMR ($CDCl_3$, TMS/ppm) δ 7.70 (m, 2H), 7.63 (m, 4H), 7.44 (m, 3H), 7.33 (m,2H), 2.99 (s, 2H), 0.50 (s, 6H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 150.4, 140.5, 140.4, 136.5, 135.0, 133.1, 128.8, 127.6, 127,5, 126,9, 125.5, 124.6, 30.5, −3.3.

2-(4-Biphenyl)-5-[dimethyl(isothiocyanatomethyl)silyl]thiophene (10b). Following the same procedure described above for the preparation of isothiocyanates **7a** and **7b**, a white solid was obtained: mp 104−105 °C; MS *m*/*e* 365 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 314 nm; IR ν_{NCS} (CCl_4) 2155 cm^{-1}; ^1H NMR ($CDCl_3$, TMS/ppm) δ 7.69 (m, 2H), 7.63 (m, 4H), 7.45 (m, 3H), 7.36(m, 1H), 7.31 (d, ^3J = 3.6 Hz), 2.53 (s, 2H), 0.55 (s, 6H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 151.1, 140.7, 140.3, 136.9, 133.4, 132.7, 132.2, 128.8, 127.6, 127.5, 126.9, 126.5, 124.7, 18.8, −2.5. Anal. Calcd for $C_{20}H_{19}SiNS_2$: C, 65.71; H, 5.24. Found: C, 65.52; H, 5.22.

5-Dimethyl(chloromethyl)silyl-2,2′:5′,2″:5″,2‴-quaterthiophene (12b). To a 3 mL toluene solution containing 0.021 mmol of $(Ph_3As)_4Pd$ prepared in situ was added 233 mg (0.41 mmol) of **12a** and 102 mg (0.41 mmol) of 5-bromo-2,2′-bithiophene. The mixture was refluxed for 6 h. After evaporation of toluene the residue was chromatographed on silica gel with petroleum ether/ethyl acetate 4:1 and then with CH_2Cl_2 as eluents. Product **19** was obtained as a yellow polycrystalline solid (85 mg, yield 48%): mp 174−175 °C; MS *m*/*e* 436 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 396 nm; ^1H NMR ($CDCl_3$, TMS/ppm) δ 7.25 (d, ^3J = 3.6 Hz), 7.23 (m, 2H), 7.18 (dd, ^3J = 3.6 Hz, ^4J = 1.2 Hz, 1H), 7.11 (d, ^3J = 3.6 Hz, 1H), 7.08 (m, 3H), 7.03 (dd, ^3J = 5.2 Hz,

^4J = 3.6 Hz, 1H), 2.96 (s, 2H), 0.48 (s, 6H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 143.1, 137.0, 136.4, 136.3, 136.2, 135.8, 135.7, 134.8, 127.9, 125.0, 124.8, 124.6, 124.4, 124.3, 124.3, 123.8, 30.4, −3.5.

5-Dimethyl(isothiocyanatomethyl)silyl-2,2′:5′,2″:5″,2‴-quaterthiophene (12c). Following the same procedure described above for the preparation of isothiocyanates **7a** and **7b**, a yellow microcrystalline solid was obtained: mp 166−167 °C; MS *m*/*e* 459 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 398 nm (ϵ = 46999); IR ν_{NCS} (CCl_4) 2155 cm^{-1}; ^{1}H NMR ($CDCl_3$, TMS/ppm) δ 7.26 (d, ^3J = 3.6 Hz), 7.23 (m, 2H), 7.18 (dd, ^3J = 3.6 Hz, ^4J = 1.2 Hz, 1H), 7.13 (d, ^3J = 3.6 Hz, 1H), 7.09 (m, 3H), 7.03 (dd, ^3J = 5.2 Hz, ^4J = 3.6 Hz, 1H), 2.51 (s, 2H), 0.53 (s, 6H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 143.9, 137.0, 136.7, 136.6, 136.5, 135.6, 135.4, 133.3 (NCS), 127.9, 125.2, 125.1, 124.6, 124.5, 124.4, 124.3, 123.8, 18.8, −2.6. Anal. Calcd for $C_{20}H_{17}SiNS_5$: C, 52.25; H, 3.73. Found: C, 52.16; H, 3.72.

5-Dimethyl(chloromethyl)silyl-3″,4″-dihexyl-2,2′:5′,2″:5″,2‴:5‴,2⁗:5⁗,2⁗-quinquethiophene-1″,1″-dioxide (13a). To a 10 mL toluene solution containing 0.01 mmol of $Pd(AsPh_3)_4$ prepared in situ was added 0.1.2 g (0.23 mmol) of **13** and 0.13 g (0.23 mmol) of **11a.** The mixture was refluxed for 8 h. Then the reaction mixture was hydrolyzed with a saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2. After the usual workup the residue was chromatographed on silica gel with petroleum ether/CH_2Cl_2 (4:1) as eluent to give 0.1 g (yield 61%) of product as red polycristalline solid: mp 99−100 °C; MS *m*/*e* 718 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 477 nm; ^{1}H NMR ($CDCl_3$, TMS/ppm) δ 7.65 (d, ^3J = 4 Hz, 1H), 7.64 (d, ^3J = 4 Hz, 1H), 7.32 (d, ^3J = 3.6 Hz, 1H), 7.26 (m, 5H), 7.06 (dd, ^3J = 5.2 Hz, 3.6 Hz, 1H), 2.99 (s, 2H), 2.68 (t, 4H), 1.56 (m, 8H), 1.38 (m, 8H), 0.93 (t, 6H), 0.48 (s, 6H);^{13}C NMR ($CDCl_3$, TMS/ppm) δ 142.3, 140.0, 139.5, 137.0, 136.8, 136.3, 136.2, 130.2, 130.1, 129.7, 128.1, 127.9, 127.4, 125.9, 125.6, 124.9, 124.7, 124.5, 31.3, 30.3, 29.6, 28.5, 27.2, 22.5, 14.0, −3.5.

5-Dimethyl(isothiocyanatomethyl)silyl-3″,4″-dihexyl-2,2′:5′,2″:5″,2‴:5‴,2^{iv}*n*-quinquethiophene-1″,1″-dioxide (13b). Following the same procedure described above for the preparation of isothiocyanates **7a** and **7b** a red polycrystalline solid was obtained: mp 80−81 °C; MS *m*/*e* 741 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 480 nm; IR ν_{NCS} (CCl_4) 2156 cm^{-1}; ^{1}H NMR ($CDCl_3$, TMS/ppm) δ 7.66 (d, ^3J = 4 Hz, 1H), 7.65 (d, ^3J = 4 Hz, 1H), 7.33 (d, ^3J = 3.6 Hz, 1H), 7.29 (dd, ^3J = 4.0 Hz, ^4J = 1.6 Hz, 1H), 7.28 (d, ^3J = 4.0 Hz, 1H), 7.26 (m, 2H), 7.24 (d, ^3J = 4.0 Hz, 1H), 7.06 (dd, ^3J = 5.2 Hz, 3.6 Hz, 1H), 2.66 (t, 4H), 2.51 (s, 2H), 1.61 (m, 8H), 1.40 (m, 8H), 0.93 (t, 6H), 0.55 (s, 6H); ^{13}C NMR ($CDCl_3$, TMS/ppm) δ 143.0, 140.1, 139.0, 137.3, 136.8, 136.7, 136.2, 134.6 (NCS), 130.3, 130.0, 129.7, 128.1, 127.4, 126.0, 125.7, 125.1, 124.7, 124.5, 31.3, 29.6, 28.5, 28.4, 27.1, 22.5, 18.6, 14.0, −2.6. Anal. Calcd for $C_{36}H_{43}SiNO_2S_6$: C, 58.26; H, 5.84. Found: C, 58.41; H, 5.86.

Conjugation of Oligothiophene Isothiocyanates with Bovine Serum Albumin. To generate the protein−thiophene conjugate, 1 mg/mL of purified bovine serum albumin, fraction V, in 0.1 M sodium carbonate (pH 9.0) as stock solution was used. The thiophene isothiocyanate was dissolved in dimethyl sulfoxide at 125 nmol/μL (stock solution). The thiophene isothiocyanate was added very slowly to the protein solution at the desired molar ratio by using 5 μL aliquots and the mixture was gently and continuously stirred during the addition. The solution was allowed to react for 48−50 h at room temperature. The protein−thiophene conjugate was separated from unbound dye by G25 Sephadex gel filtration chromatography in phosphate buffer saline (PBS) at pH 7.2.

Conjugation of Isothiocyanate 13b with anti-CD8 Antibody. Anti-CD8 antibody dissolved in phosphate buffer saline solution (pH 7.4) was dialyzed (v:v 1:1000) vs a 0.05 M carbonate buffer (pH 9.5) containing a non-ionic detergent and the dialysis was allowed to run for about 16 h at 4 °C. Then the solution was concentrated by microcon membrane (Amicon) with 10 kD cut off. Two solutions containing 2 mg/mL and two containg 4 mg/mL of anti-CD8 were prepared. To these solutions aliquots of **13b** dissolved in DMSO (10 mg/mL) were added in the amounts needed to reach protein:fluorophore 1:5, 1:10, and 1:25 molar ratios. The solutions were incubated for 48 h at room T under stirring. Afterward the conjugates were chromatographed on a desalting 1.0 mL GH25 column in PBS at pH 7.4.

Absorption and Photoluminescence Measurements. Absorption spectra were recorded with a Variant spectrophotometer. Photoluminescence measurements were performed by exciting the solutions with an He−Cd laser (λ = 325 nm) or an argon-ion laser (λ = 488 nm) and collecting the spatially averaged PL with a charge-coupled device (CCD) spectrograph. The PL quantum yields of the isothiocyanates were estimated by comparing the PL intensities of 10^{-5} M solutions in chloroform with those of the parent unsubstituted oligothiophenes at the same concentration and in the same solvent. In turn, the QYs of the parent compounds were obtained relative to a 10^{-5} M solution in chloroform of 3,5-dimethyldithieno[3,2-*b*;2′,3′-*d*]thiophene-4,4-dioxide, for which an absolute QY value of 0.77 was measured by using an integrating sphere.[18] The estimated error was ±15%.

Supporting Information Available: Synthesis and characterization of compounds **2a**, **2b**, **4a**, **4b**, **5a**, **5b**, **6a**, **6b**, **10**, **12**, **12a**, and **13** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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