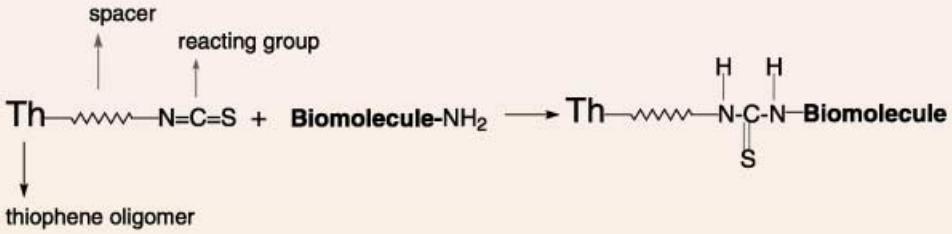
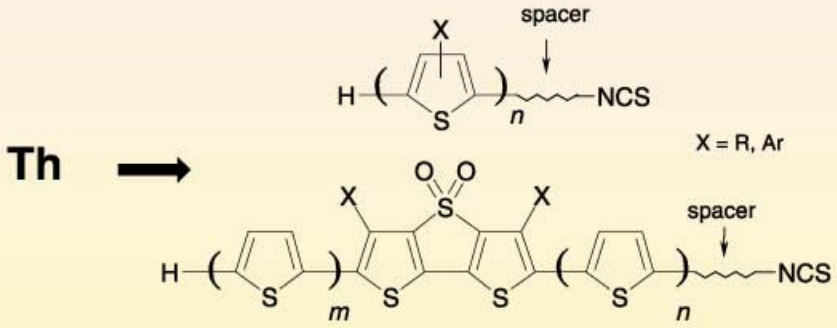
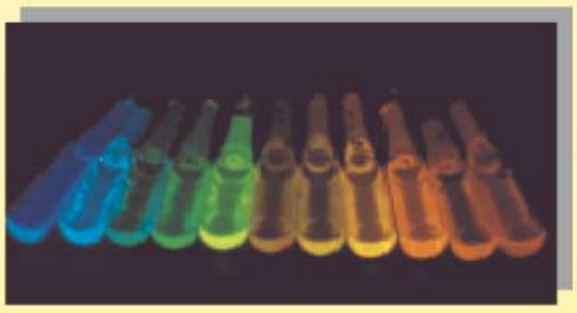
Soluble oligothiophene isothiocyanates form chemically and optically stable fluorescent conjugates with biomolecules





The emission colors of thiophene-based fluorescent markers spread across the entire visible range



0947-6539/02/0822-5072 $ 20.00+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 22

Oligothiophene Isothiocyanates as Fluorescent Markers

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Abstract: Thiophene oligomers have been studied so far mainly for their semiconductor and charge-transport properties. However, these compounds are also highly fluorescent and soluble. Solubility and fluorescence frequencies and efficiencies can be tailored by means of appropriate functionalization of the aromatic backbone. Functionalization with the isothiocyanate group (−N=C=S) allows these molecules to form covalent bonds with NH_2-containing biomolecules and give rise to optically and chemically very stable fluorescent bioconjugates. Examples of photostable conjugates formed with monoclonal antibodies are reported.

Keywords: biolabeling · fluorescence · fluorescent probes · isothiocyanates · oligothiophenes

Background

The discovery that sexithiophene is an organic semiconductor characterized by high-field-effect charge mobility dates back to 1989.[1] Since then, thiophene oligomers have enjoyed great attention mainly because of their charge-transport properties. Focusing the investigation in that direction was largely justified by the finding that the field-effect charge mobility of single-crystal sexithiophene attains values which are similar to those of amorphous silicon.[2–4]

Nevertheless, thiophene oligomers are a class of molecules characterized by another fascinating property: fluorescence. Fluorescence detection—which is a noninvasive, highly sensitive, selective, and safe technique, capable of monitoring processes even at the scale of picoseconds—is rapidly becoming the method of choice in medical and biological diagnostics, for the identification and the quantitative analysis of biomolecules, protein analysis, detection of cellular processes, immunohistochemistry, genomic studies, and nucleic acid labeling.[5–7] The use of fluorescence techniques also extends to materials science and optoelectronics. Furthermore, the growing importance of immunochemical methods for the analysis of nonextractable residues bound to the organic matrix of the soil,[8] localization and identification of pesticides,[9] or toxic compounds in water and plants[10] paves the way to the spreading of fluorescence detection techniques in environmental studies.

Synthetic difficulties and the scarce solubility of most oligothiophenes, due to their tendency to self-assemble in very stable aggregates,[11, 12] have discouraged studies of their fluorescence properties in solution, and only a few papers have been published on this topic.[12–14] More interest has been devoted to the fluorescence properties of oligothiophenes in the solid state in view of their use in thin-film electroluminescent diodes.[15–17]

Studies on the optical properties of unsubstituted oligothiophenes in solution have shown that these compounds are characterized by high absorbancies, large Stokes shifts (differences between absorption and photoluminescence frequencies), fluorescence efficiencies up to nearly 50 % (one photon re-emitted radiatively every two photons absorbed), and light emission frequencies spanning from 400 to 600 nm, on changing the oligomer size from the trimer to the hexamer.[11, 12] The same studies have also demonstrated that fluorescence lifetimes and quantum yields increase when the oligomer size is increased, and that fluorescence decay is controlled by the diffusion of localized excitons. Subsequent studies have also demonstrated that the nonradiative pathways of thiophene oligomers are largely dependent on functionalization types,[18] and that fluorescence efficiencies higher than 50 % can be attained.[19a]

All these properties—large absorbancies, large Stokes shifts, good photoluminescence quantum yields in solution, and tunability of the light emission frequencies—define a class of molecules useful as fluorescent probes, that is, as fluorophores capable of localizing within a specific region of a biological specimen and, by means of the fluorescence signal, of indicating the presence and the amount of the specimen in question.

The fluorescent markers routinely used in laboratory diagnostics are either low-weight molecules or high-weight compounds, or mixtures of compounds emitting light through a complex pattern of cascade interactions.[5–7a] The markers of different colors belong to chemically different families, require a different chemistry to bind biomolecules, and have different membrane permeabilities. The availability of a

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  0947-6539/02/0822-5073 $ 20.00+.50/0

single family of small compounds with high membrane permeability, with the same chemical structure, and fluorescing in all visible colors upon minor changes of the molecular structure itself would simplify the chemical procedures for biomolecule labeling and make multilabeling experiments—that is, experiments allowing the simultaneous monitoring of different biochemical reactions and cellular events—more accessible. Currently, there is great demand for new fluorophores with better spectral properties, greater tunability of emission frequencies, and that are easily synthesized, and allow the standardization of the procedures for binding biomolecules.

Thiophene oligomers emit light across the entire visible range and are very stable and easy to functionalize. Moreover, they are soluble in most organic solvents and even in water.[19b] Their use as fluorescent markers would allow the standardization of the procedures for binding the molecules to probe.

The Challenge

What was lacking at the end of the 90s, despite the huge number of papers devoted to the chemistry of thiophene and thiophene oligomers,[20–23] was a functionalization type which would not alter the useful optical properties and at the same time would allow the covalent binding of oligomers of different sizes to the molecules of interest. For biological molecules, the best candidate for this purpose was the isothiocyanate group, which spontaneously reacts with the ε-NH_2 groups of the lysine residues to form ureas ([Eq. (1)]; X = fluorescent marker, Y = biological molecule).

$$X{-}NCS + Y{-}NH_2 \rightarrow X{-}NH{-}C(S){-}NH{-}Y \quad (1)$$

Furthermore, other functional groups of biological molecules of great interest can be transformed into the amino group by chemical synthesis, such as, for example, oligodeoxynucleotides at the 5′ end position.[5] Thus, oligothiophenes containing the isothiocyanate functionality could become useful to monitor a variety of processes of biological importance.

Therefore, the challenge was to demonstrate that it was possible: 1) to synthesize fluorescent oligothiophene isothiocyanates; 2) to bind them to biomolecules and to get chemically stable bioconjugates; 3) to prove that binding to biomolecules did not lead to the quenching of fluorescence, as in the case of many fluorescent organic molecules.

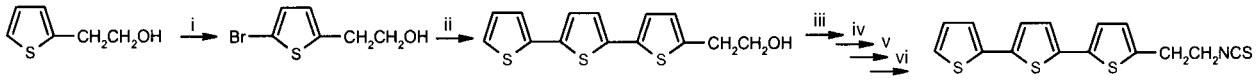
Synthesis and Optical Properties of Oligothiophene Isothiocyanates

Two different patterns were developed in our laboratory for introducing the isothiocyanate functionality into the molecular backbone of thiophene-based oligomers, which are illustrated in Schemes 1 and 2.

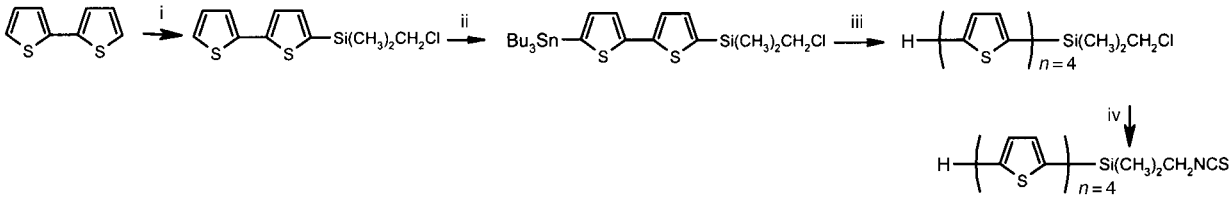
The first pattern takes advantage of the availability of commercial thiophenes functionalized with the hydroxyl functionality. Through palladium-catalyzed cross-coupling reactions,[24] these derivatives can be used to build thiophene oligomers of variable sizes, containing the OH functionality either at one of the β positions or at one of the terminal α positions. The hydroxyl functionality can be transformed into the isothiocyanate group through the sequence: alcohol →tosylate →azide →amine →isothiocyanate, as shown in Scheme 1 for the trimer prepared starting from commercial 2-(2-thienyl)ethanol.[25]

The second pattern takes advantage of the commercial availability of chloro(chloromethyl)dimethylsilane, $Cl{-}CH_2Si(CH_3)_2Cl$ (Scheme 2). Since the chlorine atom attached to silicon is more reactive than the chlorine atom attached to carbon, the $Cl{-}CH_2{-}Si(CH_3)_2{-}$ group can be directly linked to one of the terminal positions of a thiophene oligomer by lithiation followed by reaction with chloro(chloromethyl)dimethylsilane. Afterwards, the chlorine attached to carbon can be transformed into the corresponding isothiocyanate by reaction with sodium thiocyanate NaCNS.[25]

As an alternative, a thiophene ring functionalized with the $-Si(CH_3)_2CH_2Cl$ moiety can be coupled by the Stille reac-



Scheme 1. Synthetic pattern for the synthesis of the terthiophene isothiocyanate prepared starting from commercial 2-(2-thienyl)ethanol: i) *N*-bromosuccinimide, toluene, −20 °C; ii) 5-tributylstannyl-2,2′-bithiophene, $[Pd(AsPh_3)_4]$, 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.



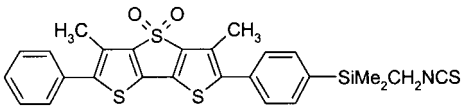
Scheme 2. An alternative pattern for the synthesis of quaterthiophene isothiocyanate with the aid of commercially available chloro(chloromethyl)dimethylsilane: i) Lithium diisopropylamide, $ClCH_2Me_2SiCl$; ii) *n*BuLi, $[(Bu)_3SnCl]$; iii) 5-bromo-2,2′-bithiophene, $[Pd(AsPh_3)_4]$, toluene, 110 °C; iv) NaSCN, acetone, Et_2O.

0947-6539/02/0822-5074 $ 20.00+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 22

tion[24a] to an oligomer of variable size. This methodology allows the insertion of the NCS functionality at the last step of the procedure.[25] Recently, we have obtained in a similar way thiophene oligomers with a terminal phenyl group functionalized with the $-Si(CH_3)_2CH_2NCS$ moiety.[26]

We were able to prepare a series of oligothiophene isothiocyanates,[25–27] which emit light across the entire visible range, with fluorescence quantum yields ranging from a few percent to 90–100%. The optical characteristics of a few of these compounds have already been reported.[25] To have an idea of how many fluorescence colors can be obtained with thiophene oligomers (see the picture reported in the frontispiece), a real color picture was taken under irradiation *with a single light source*, a UV lamp exciting at $\lambda = 364$ nm.

Scheme 3 shows the structure and the optical characteristics of a thienylene/phenylene-based isothiocyanate (DTTPP)[26] containing the "rigid-core" 3,5-dimethyl-dithieno[3,2-b;2′,3′-d]thiophene-4,4-dioxide. Oligothiophenes containing the rigid dithienothiophene *S*,*S*-dioxide moiety are characterized by high fluorescence efficiencies both in solution and in the solid state.[19a] As shown in Scheme 3, DTTPP attains a fluorescence quantum yield which is greater than 90% (almost every absorbed photon is re-emitted radiatively).



Scheme 3. Molecular structure and optical characteristics of 3,5-dimethyl-2,6-diphenyl-dithieno[3,2-b;2′,3′-d]thiophene-4,4-dioxide (DTTPP). ($\lambda_{max}(CH_2Cl_2) = 404$ nm; $\lambda_{PL}(CH_2Cl_2) = 499$ nm; $\varepsilon = 23\,372$ cm^{-1}M^{-1}; QY > 90% QY is the photoluminescence quantum yield).

Figure 1 shows the normalized absorption and photoluminescence spectrum of a 10^{-6}M solution of DTTPP in methylene chloride, characterized by quite a large Stokes shift (4712 cm^{-1}). The optical characteristics of DTTPP fall in the range of those of fluorophores of current practical interest, the molar extinction coefficients of which are in the range 5000–200 000 cm^{-1}M^{-1} and the photoluminescence quantum yields in the range 5 to 100%.[5] A solution of DTTPP stored at room temperature showed unaltered optical characteristics after five months.

Several chemically and optically very stable oligothiophene isothiocyanates, containing conventional thiophene rings and/or thiophene-*S*,*S*-dioxide or the dithienothiophene *S*,*S*-dioxide moieties, have been synthesized in our laboratory, with molar extinction coefficients ranging from 8000 to 47 000 cm^{-1}M^{-1} and fluorescence quantum yields up to >90%. For some of these compounds the absolute fluorescence quantum yields in solution were measured with the use of an integrating sphere. In this way we have identified a series of standards for fluorescence frequencies in the blue, green, yellow, orange, and red regions to use for the monitoring of the relative fluorescence quantum yields of newly synthesized oligothiophene isothiocyanates.

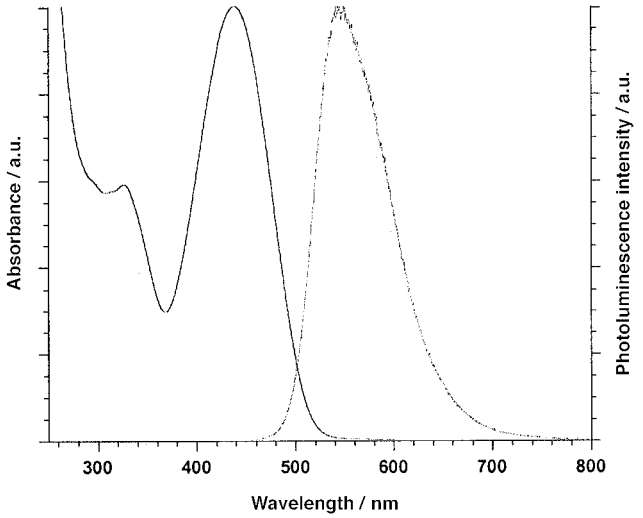


Figure 1. Absorption (left) and photoluminescence (right) spectrum of a 10^{-6}M solution of DTTPP in methylene chloride.

The Conjugation of Thiophene-Based Fluorophores to Proteins and the Optical Characterization of the Bioconjugates

The conjugation of oligothiophene isothiocyanates to biomolecules occurs spontaneously at basic pH. We have already reported the results of the conjugation of thiophene-based fluorophores with bovine serum albumin (BSA, fraction V) and the monoclonal antibody anti-CD8.[25] BSA is the cheapest commercially available protein, while anti-CD8 is an IgG1 isotype-specific antibody, which is commonly used for diagnostic purposes in flow cytometry and immunohistochemical applications.[28, 29]

The conjugation of oligothiophene isothiocyanates with BSA and antibody anti-CD8 was carried out according to standard methods.[25] The oligothiophene–protein conjugates were separated from unbound isothiocyanate by gel filtration chromatography in saline phosphate buffer solution (PBS, pH 7.2).

The conjugation did not lead to the quenching of the fluorescence signal of the isothiocyanate. Thus, a series of experiments were carried out in order to evaluate: 1) the maximum isothiocyanate to protein molar ratio which could be attained without protein precipitation; 2) the optimum reaction time; 3) the optical stability of the bioconjugate.

Values of the fluorophore to protein molar ratios up to 30:1 were attained after a few hours of mixing the solutions containing the isothiocyanate and the protein, without precipitation of the conjugate. The intensities of the photoluminescence spectrum of the conjugates showed that the signal intensity increased as the isothiocyanate/protein molar ratio increased. The photoluminescence spectrum of the conjugates versus the exposure to UV irradiation showed also a very good response to irradiation power and irradiation time. For example, the maximum intensity of the PL spectrum of the conjugate with BSA was still more than half the starting value after 45 hours of continuous exposure to laser irradiation.[25]

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Figures 2 and 3 show the absorption and photoluminescence spectra of the conjugate of DTTPP with antibody anti-CD3, obtained according to standard methods. The anti-CD3 is a IgG1 isotype-specific antibody (22–30 kDa molecular weight, purified from mouse ascitic fluid), which is directed against the CD3-antigen expressed on human T cells and used for identification of normal and malignant T lymphocytes.

In Figure 2 the absorption spectrum of free DTTPP is compared with that of DTTPP bound to antibody anti-CD3. The figure shows that the absorption spectrum of the fluorophore remains unaltered after conjugation, except for some broadening of the low-energy absorption band, probably due to the increase in the number of rotational conformers.

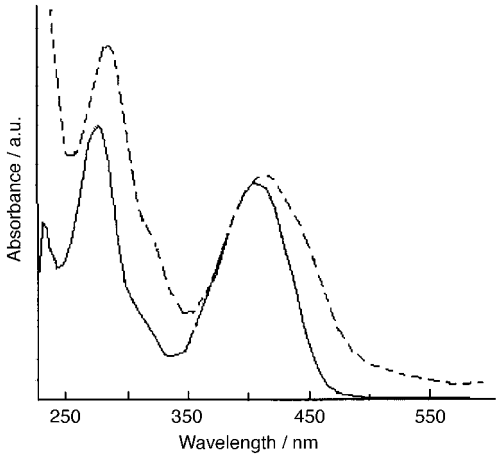

Figure 2. Absorption spectrum of a 10^{-6} M solution of free DTTPP in DMSO (——) and of its conjugate with the anti-CD3 antibody (- - - -).

Irradiation at $\lambda_{exc} = 404$ nm leads to an intense photoluminesce signal, as shown in Figure 3, which remains still of sizeable intensity when the sample is irradiated at $\lambda_{exc} =$ 480 nm. The significance of this result stems from the fact that 480 nm is the wavelength of the argon-ion laser excitation source of the currently available commercial flow cytometers.

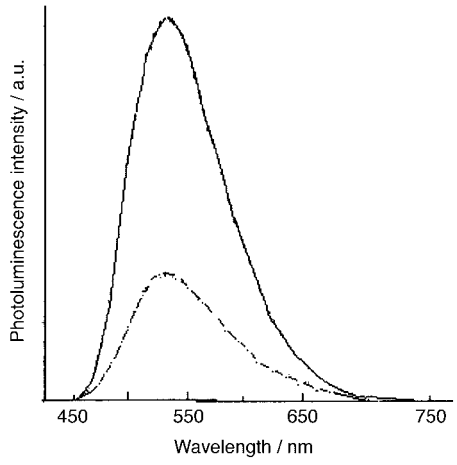

Figure 3. Photoluminescence spectrum of the DTTPP/anti-CD3 conjugate under irradiation at $\lambda_{exc} = 404$ nm (——) and at $\lambda_{exc} = 480$ nm (•–•–).

Both in the case of the conjugate with antibody anti-CD8 and antibody anti-CD3 it was found that the activity of the antibody was completely preserved in the conjugate. The solutions were tested systematically for a period of a few months and found to be unaltered.

In summary, our data show that it is possible to regioselectively functionalize thiophene oligomers with the isothiocyanate group; that oligothiophene isothiocyanates are photoluminescent compounds, the emission frequencies of which can be tuned across the visible range from blue to red; that they spontaneously bind to proteins and produce photoluminescent bioconjugates characterized by high photostability and chemical stability; that the bioconjugates can undergo repeated excitation/emission cycles without changes in fluorescence signal, unlike conventional dyes such as fluorescein. Being uncharged, oligothiophene isothiocyanates are pH-insensitive probes. As they are low-weight molecules (less than 1 kDa molecular weight), they should also be more membrane-permeant than many currently used fluorophores and be more useful for analyses with living cells.

Future Challenges and Perspectives

There is much room for exploitation of thiophene oligomers as fluorescent markers, both in basic research and in industrial applications. The synthetic methodology developed so far for the introduction of the isothiocyanate group is flexible and allows for the preparation of isothiocyanates with spacers of variable lengths or containing the NCS group at both ends of the molecule. More importantly, the great versatility of thiophene chemistry allows for the functionalization of thiophene oligomers with many other functional groups capable of binding proteins or DNA components, vitamins, hormones, and so on,[5] and this greatly increases the range of applicability of the new class of fluorescent probes.

The great optical stability of oligothiophene-based fluorophores will make them very useful as tracers in a variety of new experiments. Moreover, their optical stability will allow detailed studies of their optical properties as a function of oligomer size, backbone functionalization, and solvent properties, as well as studies of fluorescence lifetimes and nonradiative pathways for energy relaxation from the excited state.

With respect to other systems characterized by tunable fluorescence frequencies and high fluorescence quantum yields,[30] the synthesis of oligothiophene isothiocyanates is much simpler and safe, and the conjugation procedures with biomolecules are much easier to carry out by standard methods.

My research group is currently actively involved in investigations aimed at making the new class of fluorophores suitable for the market of biological or other screening operations and have started spin-off work aimed at bridging the gap between basic research and the requirements for industrial applications. In particular, an attempt has been made to demonstrate the feasibility of multilabeling experiments, which would allow the simultaneous use of a few of our fluorescent probes for the measurement of different cellular events, as this is crucial for future applications in clinical tests.

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Acknowledgments

Thanks are due to M. Zambianchi (CNR-ISOF, Bologna) for the picture reported in the frontispiece, to G. Sotgiu (CNR-ISOF, Bologna) for the synthesis of DTTPP, to A. Ventola (BIO D srl, Bari) for the conjugation experiments of DTTPP with antibody anti-CD3, and to Dr. G. Gigli, University of Lecce, for setting up the apparatus and measuring fluorescence quantum yields for the isothiocyanates.

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