

Supporting Information

for

Human Serum Albumin-Oligothiophene Bioconjugate: A Phototheranostic Platform for Localized Killing of Cancer Cells by Precise Light Activation

Andrea Cantelli,¹ Marco Malferrari,¹ Alice Soldà,¹ Giorgia Simonetti,² Sonny Forni,¹ Edoardo Toscanella,¹ Edoardo J. Mattioli,¹ Francesco Zerbetto,¹ Alberto Zanelli,³ Matteo Di Giosia,¹ Mattia Zangoli,^{3,4} Giovanna Barbarella,^{3,4} Stefania Rapino^{,1} Francesca Di Maria^{*,3,4} Matteo Calvaresi^{*,1}*

¹ Dipartimento di Chimica “Giacomo Ciamician” - Alma Mater Studiorum – Università di Bologna
Via Francesco Selmi, 2, 40126 Bologna, Italy

² IRCCS Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori", Via Piero Maroncelli, 40, 47014 Meldola, FC, Italy

³ Istituto per la Sintesi Organica e la Fotoreattività (ISO-F), Consiglio Nazionale delle Ricerche, Via Piero Gobetti, 101, 40129 Bologna, Italy

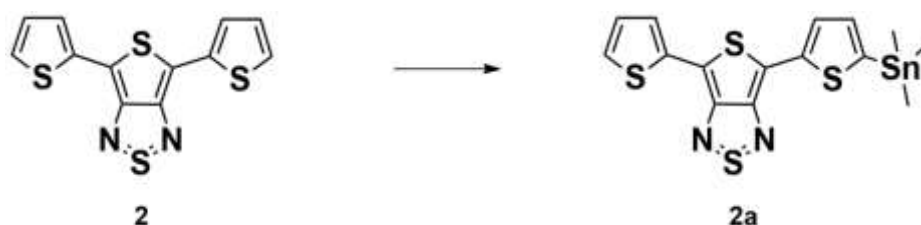
⁴ Mediteknology srl, Via Piero Gobetti, 101, 40129 Bologna, Italy

Oligothiophene N-hydroxysuccinimidyl esters synthesis

Compounds 2,5-dioxopyrrolidin-1-yl-5'-(7-(thiophen-2-yl)benzo[c][1,2,5]thiadiazol-4-yl)2-2'-bithiophene-5-carboxylate (**3a**), 5,7-di(thiophen-2-yl)thieno[3,4-b]pyrazine (**1**) and 5,7-di(thiophen-2-yl)thieno[3,4-c]-[1,2,5]thiadiazole (**2**) were prepared according to the procedures reported in the literature.^[1-4]

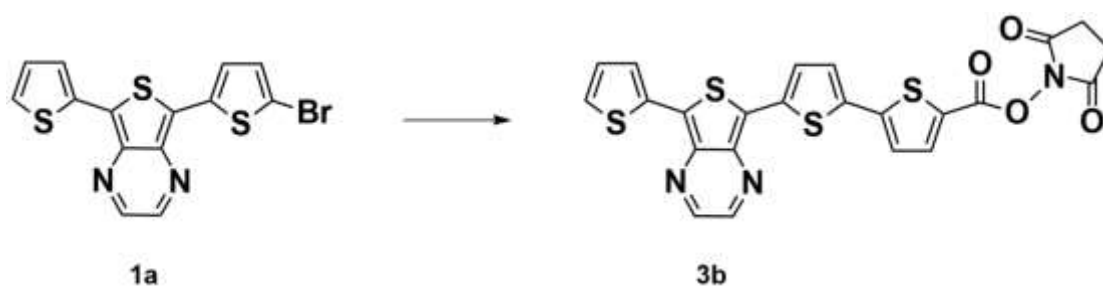


5-(5-bromothiophen-2-yl)-7-(thiophen-2-yl)thieno[3,4-b]pyrazine (1a): To a solution of 5,7-di(thiophen-2-yl)thieno[3,4-b]pyrazine (**1**) (197 mg, 0.65 mmol) in CH₂Cl₂/CH₃COOH (20 mL, 8:2 v/v), NBS (93 mg, 0.52 mmol) was added. The reaction mixture was sonicated for 20 min at room temperature. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography (CH₂Cl₂:Cyclohexane 40:60 v/v). Yield 60 %. Deep purple powder. EI-MS *m/z* 379 (M⁺). ¹H-NMR (400 MHz, CDCl₃): δ 8.54 (s, 1H), 8.52 (s, 1H), 7.66 (d, J = 3.2 Hz, 1H), 7.43 (d, J = 4 Hz, 1H), 7.32 (d, J = 3.2 Hz, 1H), 7.14 (dd, ³J = 4 Hz, ³J = 3.2 Hz, 1H), 7.08 (d, J = 3.2, 1H).

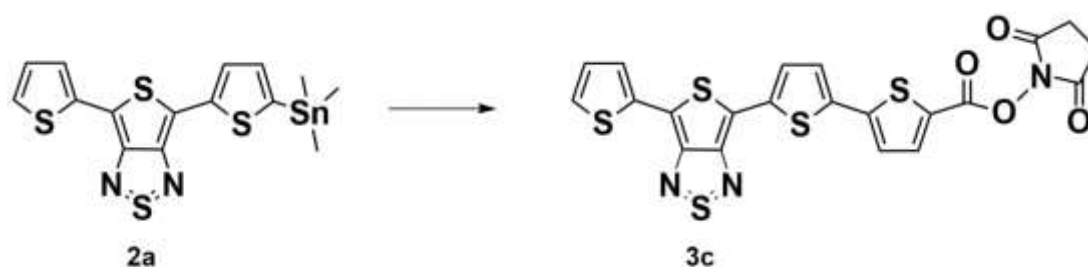


5-(thiophen-2-yl)-7-(5-(trimethylstannyl)thiophen-2-yl) thieno[3,4-c]-[1,2,5]thiadiazole (2a). To a stirred solution of 2,2,6,6-tetramethylpiperidine, TMP, (120 mg, 0.85 mmol) in dry THF (5 mL) at 0° C, a 2.5 M solution of *n*-BuLi in hexane (0.85 mmol) was rapidly added. The mixture was kept

to react at this temperature for 15 minutes, and then a solution of **2** (200 mg, 0.65 mmol) in dry THF was slowly added by a syringe. The resulting mixture was allowed to warm to room temperature and stirred overnight before quenching with water. The product was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous sodium sulphate and the solvent was removed under reduced pressure. ¹H NMR of the crude product showed a mixture of **2a/2** in the ratio 40:60. The crude product was utilized as obtained for the last synthetic step. ¹H-NMR (400 MHz, CDCl₃): δ 7.63 (d, J = 3.6 Hz, 1H), 7.54 (d, J = 4 Hz, 1H), 7.30 (d, J = 5.2 Hz, 1H), 7.17 (d, J = 3.6 Hz, 1H), 7.14 (dd, ³J = 5.2 Hz, ³J = 3.6 Hz, 1H), 7.08 (d, J = 3.2, 1H), 0.42 (s, 9H).



2,5-dioxopyrrolidin-1-yl 5'-((7-(thiophen-2-yl)thieno[3,4-b]pyrazin-5-yl)-[2,2'-bithiophene]-5-carboxylate (3b). A mixture of **1a**, (241 mg, 0.63 mmol), 2,5-dioxopyrrolidin-1-yl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)thiophene-2-carboxylate prepared according to ref. 1 (331 mg, 0.94 mmol), Pd(dppf)Cl₂ (51 mg, 0.1 mmol) and NaHCO₃ (78 mg, 0.93 mmol) in THF/H₂O (2:1, 3 mL) was irradiated with microwaves at 80°C for 25 min. The reaction mixture was brought to room temperature and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (CH₂Cl₂:AcOEt:Cyclohexane 30:10:60 v/v). Yield 63 %. Deep purple powder. EI-MS *m/z* 523 (M⁺). ¹H-NMR (400 MHz, CDCl₃): δ 8.58 (s, 2H), 7.95 (d, J = 4.0 Hz, 1H), 7.69 (d, J = 3.6 Hz, 1H), 7.57 (d, J = 4.0 Hz, 1H), 7.46 (d, J = 5.2 Hz, 1H), 7.37 (d, J = 4.0 Hz, 1H), 7.32 (d, J = 4.0 Hz, 1H), 7.16 (dd, J = 5.2 Hz J = 3.6 Hz, 1H), 2.92 (s, 4H).



2,5-dioxopyrrolidin-1-yl 5'-(7-(thiophen-2-yl)thieno[3,4-c]-[1,2,5]thiadiazole)-[2,2'-bithiophene]-5-carboxylate (3c). A mixture of 2,5-dioxopyrrolidin-1-yl 5-bromothiophene-2-carboxylate prepared according to ref. 1 (65 mg, 0.21 mmol), **2a** (100 mg, 0.21 mmol), Pd(PPh₃)₄ (12 mg, 0.05 mmol) in Toluene (10 mL) was refluxed overnight. The reaction mixture was brought to room temperature and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (CH₂Cl₂:AcOEt:Cyclohexane 30:10:60 v/v). Yield 51 %. Dark green powder. EI-MS *m/z* 529 (M⁺). ¹H-NMR (400 MHz, CDCl₃): δ 7.93 (d, *J* = 4.0 Hz, 1H), 7.62 (d, *J* = 3.6 Hz, 1H), 7.49 (d, *J* = 4 Hz, 1H), 7.37 (d, *J* = 5.2 Hz, 1H), 7.33 (d, *J* = 4.0 Hz, 1H), 7.26 (d, *J* = 4.0 Hz, 1H), 7.13 (dd, *J* = 5.2 Hz *J* = 3.6 Hz, 1H), 2.91 (s, 4H).

Oligothiophene N-hydroxysuccinimidyl esters characterization

^1H NMR and Mass spectra

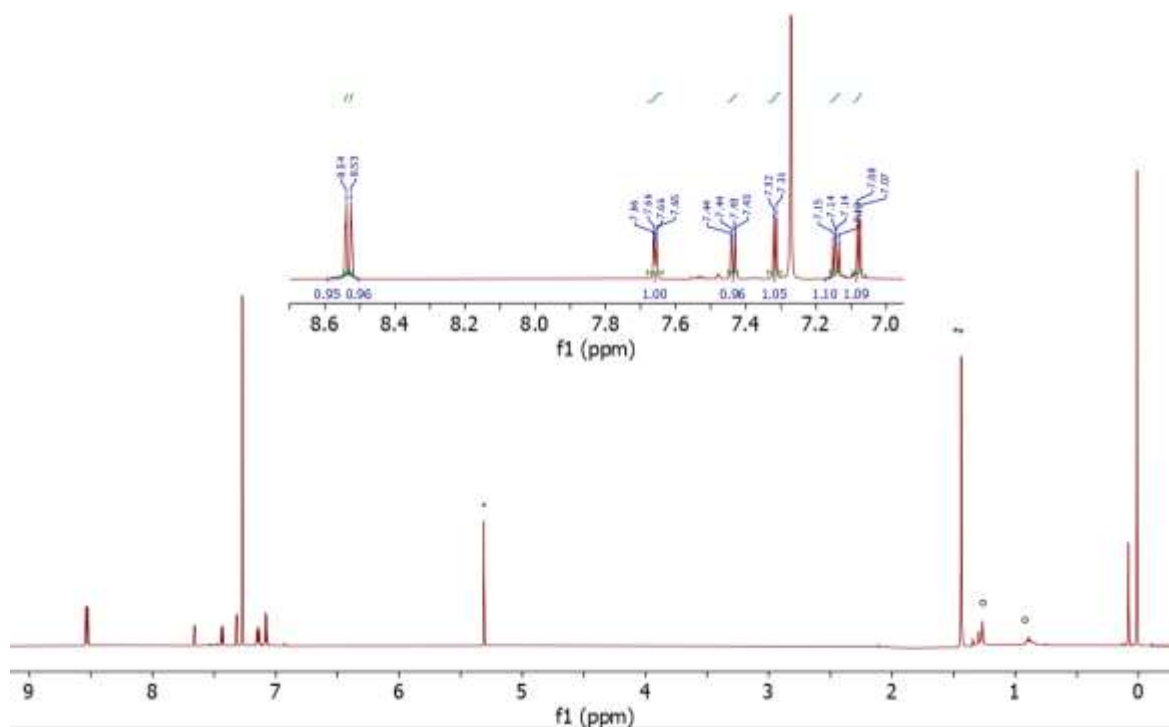


Figure S1. ^1H NMR spectrum of compound **1a** (* = CH_2Cl_2 , ** = Cyclohexane, ° = Grease).

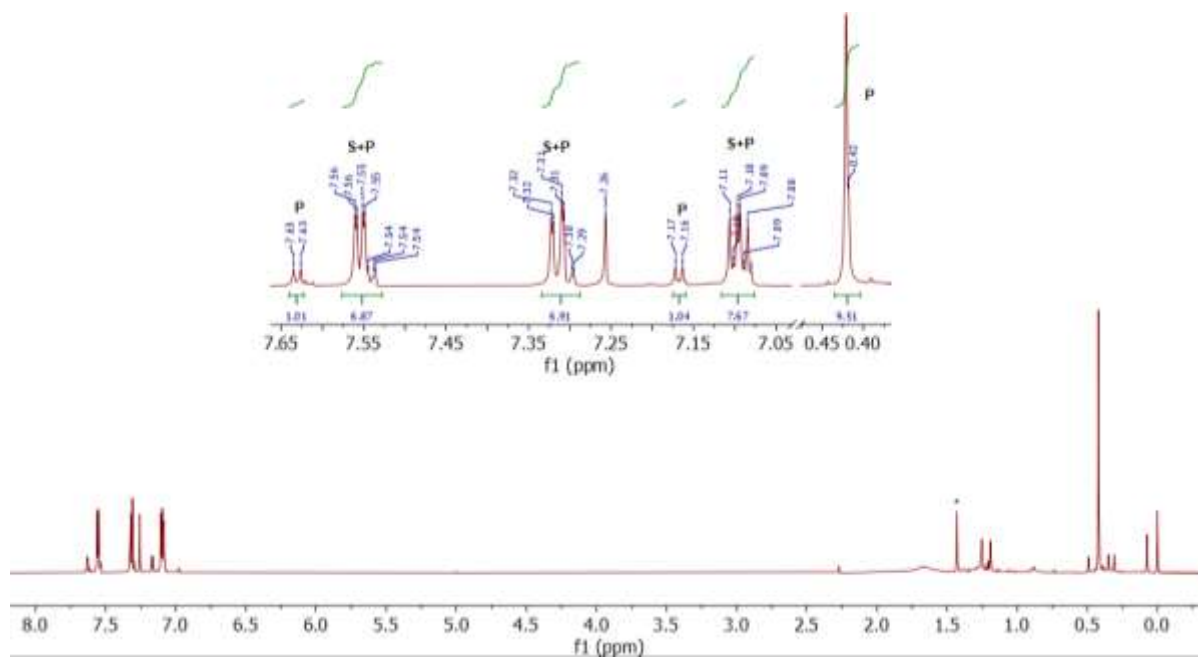


Figure S2. ^1H NMR spectrum of compound **2a** (* = Cyclohexane, ° = Grease, S = Starting material, P = Product).

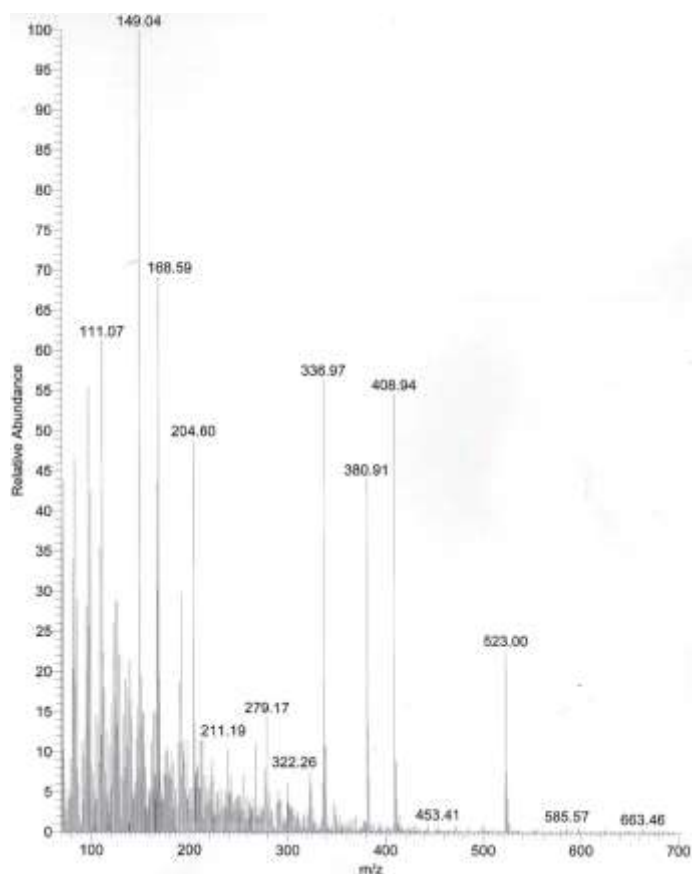
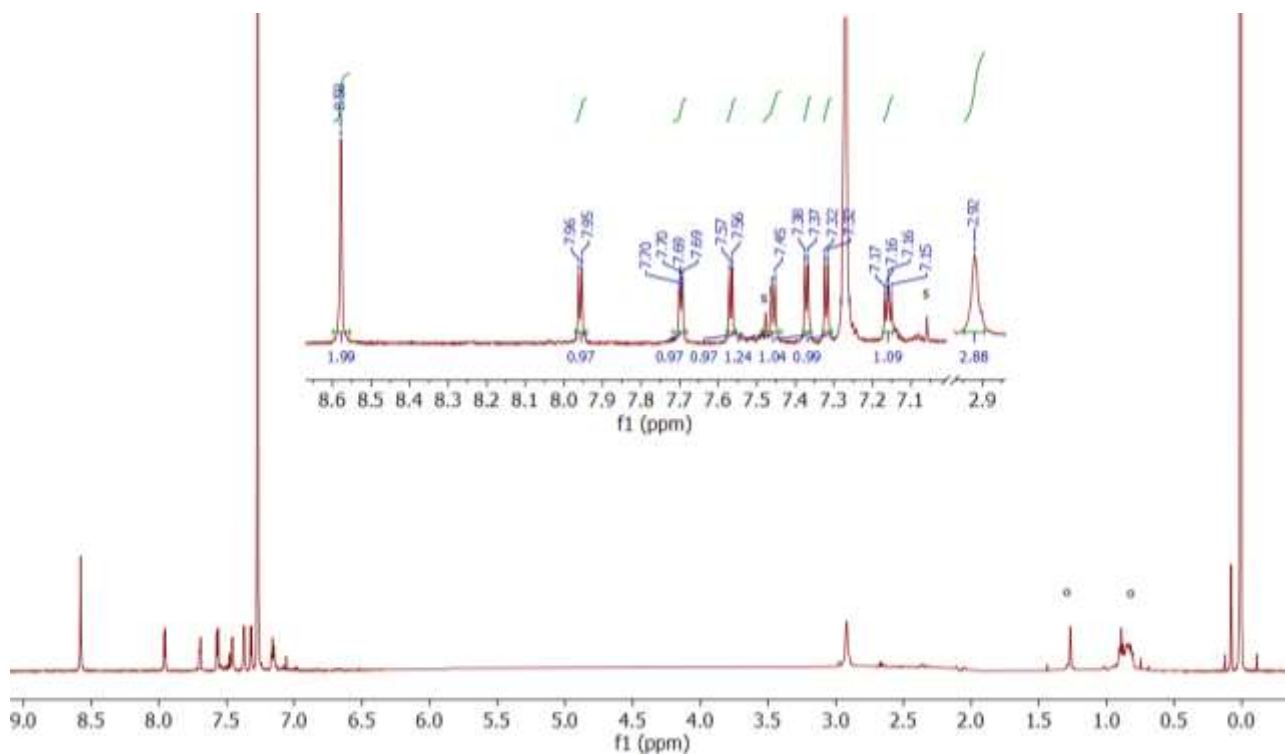


Figure S3. $^1\text{H-NMR}$ (s = Side bands, o = Grease) and Mass spectrum of compound **3b**.

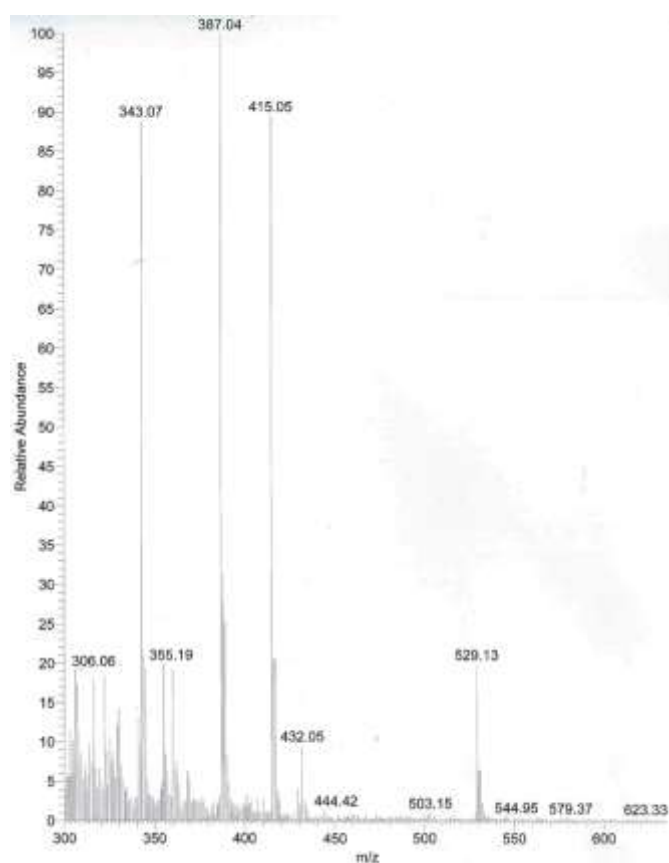
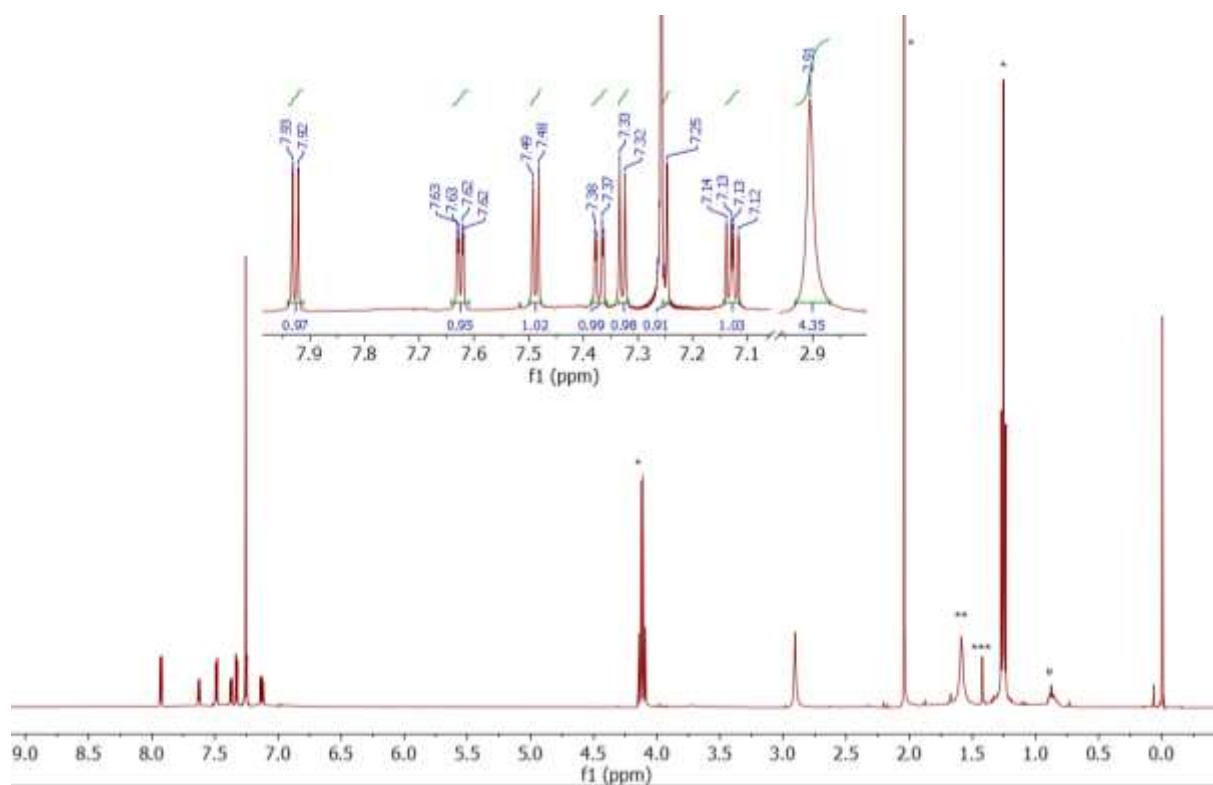


Figure S4. ^1H NMR (* = AcOEt ** = H_2O *** = Cyclohexane, ° = Grease) and Mass spectrum of compound **3c**.

Cyclic Voltammetry measurements

Experimental

Cyclic voltammetries were performed at scan rates from 20 to 200 $\text{mV}\cdot\text{s}^{-1}$ in a three-compartment glass electrochemical cell at room temperature under Ar pressure, by using an AMEL electrochemical system model 5000. The electrolytic solution was CH_2Cl_2 (Merck, Uvasol, distilled over P_2O_5 and stored under Ar pressure) with $0.1 \text{ mol}\cdot\text{L}^{-1}$ $(\text{C}_4\text{H}_9)_4\text{NClO}_4$ (Fluka, puriss. crystallized from CH_3OH and vacuum dried) purged with Ar just before the measurements. Working electrode was Pt disk (diameter 1 mm), reference electrode was aqueous KCl Saturated Calomel Electrode ($\text{SCE} = 0,47 \text{ V}$ versus ferrocene/ferricinium in CH_2Cl_2 $0.1 \text{ mol}\cdot\text{L}^{-1}$ $(\text{C}_4\text{H}_9)_4\text{NClO}_4$) and auxiliary electrode was a Pt wire. HOMO (E_{HOMO}), LUMO (E_{LUMO}) and band gap (E_{g}) energies were estimated on the basis of the following relationships: $E_{\text{HOMO}} = - (E_{\text{ox}} + 4.68)$, $E_{\text{LUMO}} = - (E_{\text{red}} + 4.68)$, $E_{\text{g}} = - (E_{\text{HOMO}} - E_{\text{LUMO}})$. Calculated values are expressed in eV units.

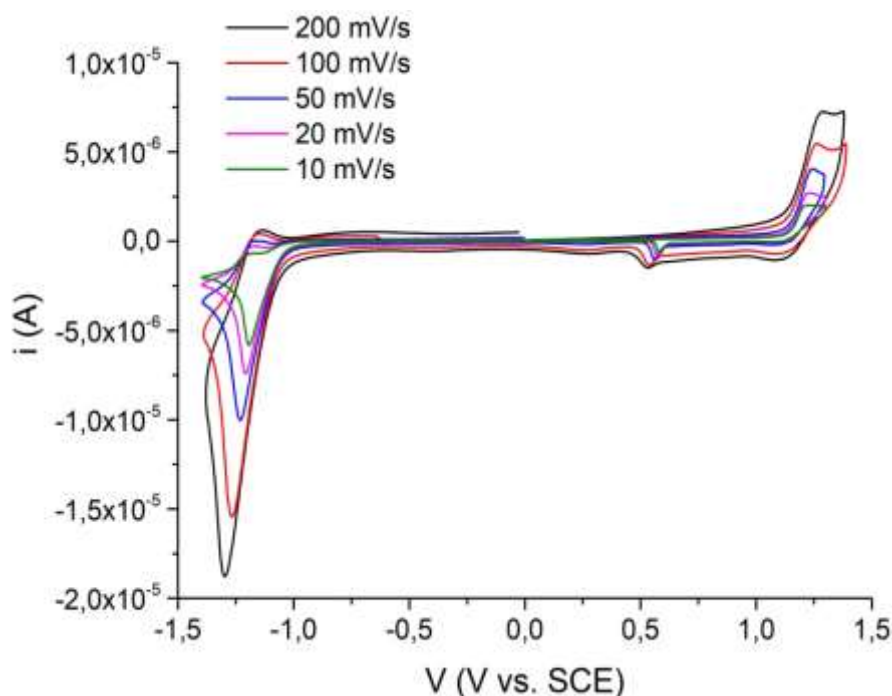


Figure S5. Cyclic voltammetries of **3a** 1 mM in CH_2Cl_2 0.1 M TBAP

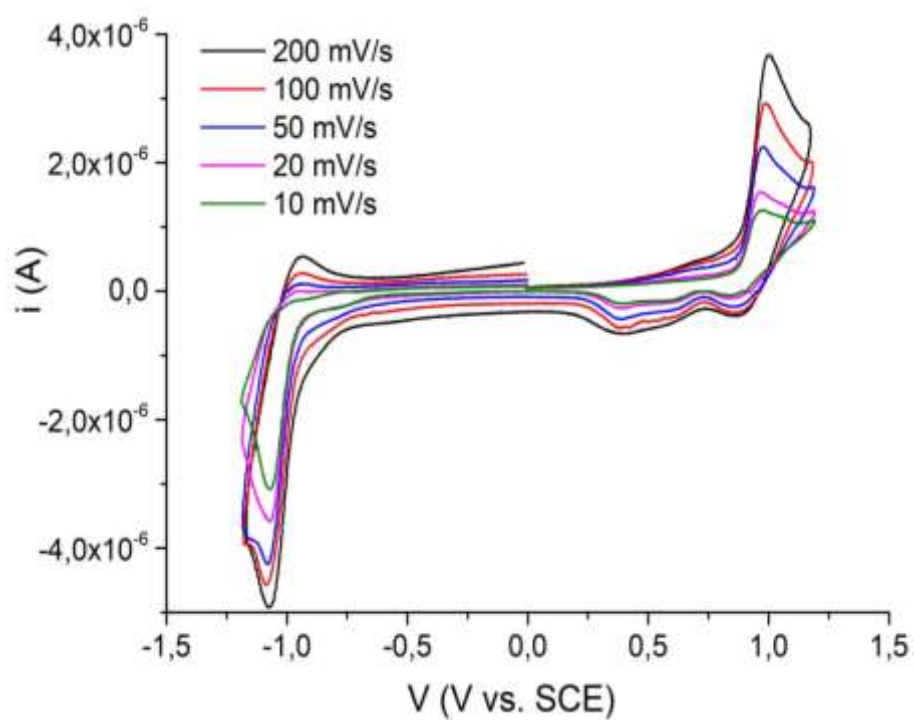


Figure S6. Cyclic voltammeteries of **3b** 1 mM in CH₂Cl₂ 0.1 M TBAP

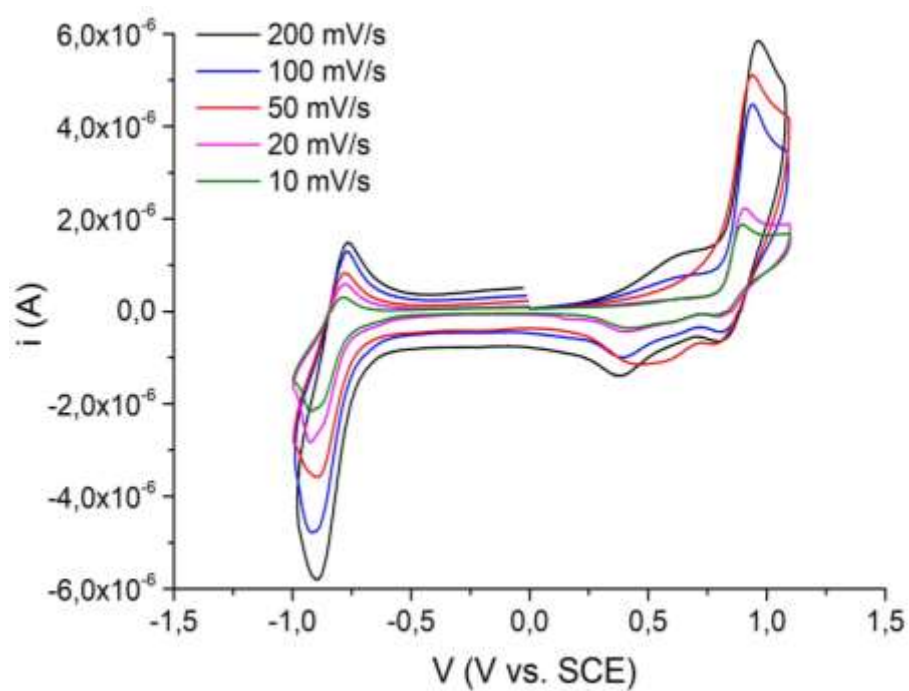


Figure S7. Cyclic voltammeteries of **3c** 1 mM in CH₂Cl₂ 0.1 M TBAP

Figures S5, S6 and S7 show the typical decrease in peak current with scan rate. The higher peak current in the oxidation wave of **3a**, compared to those of **3b** and **3c**, is reasonably due to the combination of three factors: i) uncertainly about the concentration of the molecule; ii) small differences in diffusion coefficients; iii) small differences in kinetic constants. On the other hand, the peak current of the reduction wave of **3a** is twice that of the oxidation of the same molecule, and more than twice that of the reduction of **3b** and **3c**, consequently it can be argued that the reduction process is bielecronic.

Table S1. Summary of the electrochemical properties.

Sample	E_{ox}	E_{red}	HOMO	LUMO	E_g
3a	1.20	-1.31	5.84	3.37	2.47
3b	0.93	-1.01	5.60	3.67	1.93
3c	0.88	-0.85	5.58	3.83	1.75

Materials

Human Serum Albumin (HSA) (Cat. No. A3782), 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red) (Cat. No. 90101), Type VI-A Peroxidase from horseradish lyophilized powder (HRP) (Cat. no. P6782), Hydrogen Peroxide Solution 30% (w/w) (Cat. No. 31642-M), 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABMDMA) (Cat. No. 75068), Agarose for Molecular Biology (Cat. No. A9539), Glycerol (Cat. No. G2025), Comassie Brilliant Blu (Cat. No. 27815), Deuterium Oxide (Cat. No. 151882-100G), Amicon Ultra centrifugal filters MWCO 30 kDa Millipore, UFC503024 (Cat. No. Z677892-24EA), Dimethyl Sulfoxide (DMSO) (Cat. No. 472301), N,N-dimethylformamide (DMF) (Cat. No. 227056), Sodium Chloride (Cat. No. S9888-M), Potassium phosphate monobasic (Cat. No. P0662-M), Sodium phosphate dibasic (Cat. No. S0876), Potassium chloride (Cat. No. P3911M), Sodium bicarbonate (Cat. No. 31437-M), Sodium carbonate (Cat. No. 223530), 14 kDa cellulose dialysis tubing (Cat. No. D9652) were purchased from Sigma Aldrich (Merck). Milli-Q water was used for the preparation of all the aqueous solutions.

HSA-Oligothiophene Characterization

Photophysical Characterization

UV-vis absorption spectra were recorded using a Cary 60 UV-Vis spectrophotometer (Agilent).

Luminescence spectra and fluorescence quantum yields measurements were performed with an Edinburgh Analytical Instruments FLS920, equipped with a time-correlated single-photon counting device for luminescence lifetimes measurements, a nitrogen cooled EO-817S Germanium Detector system with a Ge PIN diode detector for IR emission spectra acquisitions and Glan-Thompson polarizers for anisotropy determinations.

Emission spectra and fluorescence quantum yields (QY) were measured in PBS 10 mM for the protein conjugates and in DMF for the unconjugated fluorophores. Sample concentrations were adjusted to present an absorbance of 0.01 at the wavelength corresponding to the lowest energy transition of each compound. The same wavelength was used for the excitation of a iso-absorbing solutions of Tris(bipyridine)ruthenium(II) chloride $\text{Ru}(\text{bpy})_3\text{Cl}_2$, used as reference standards (QY = 0.28).

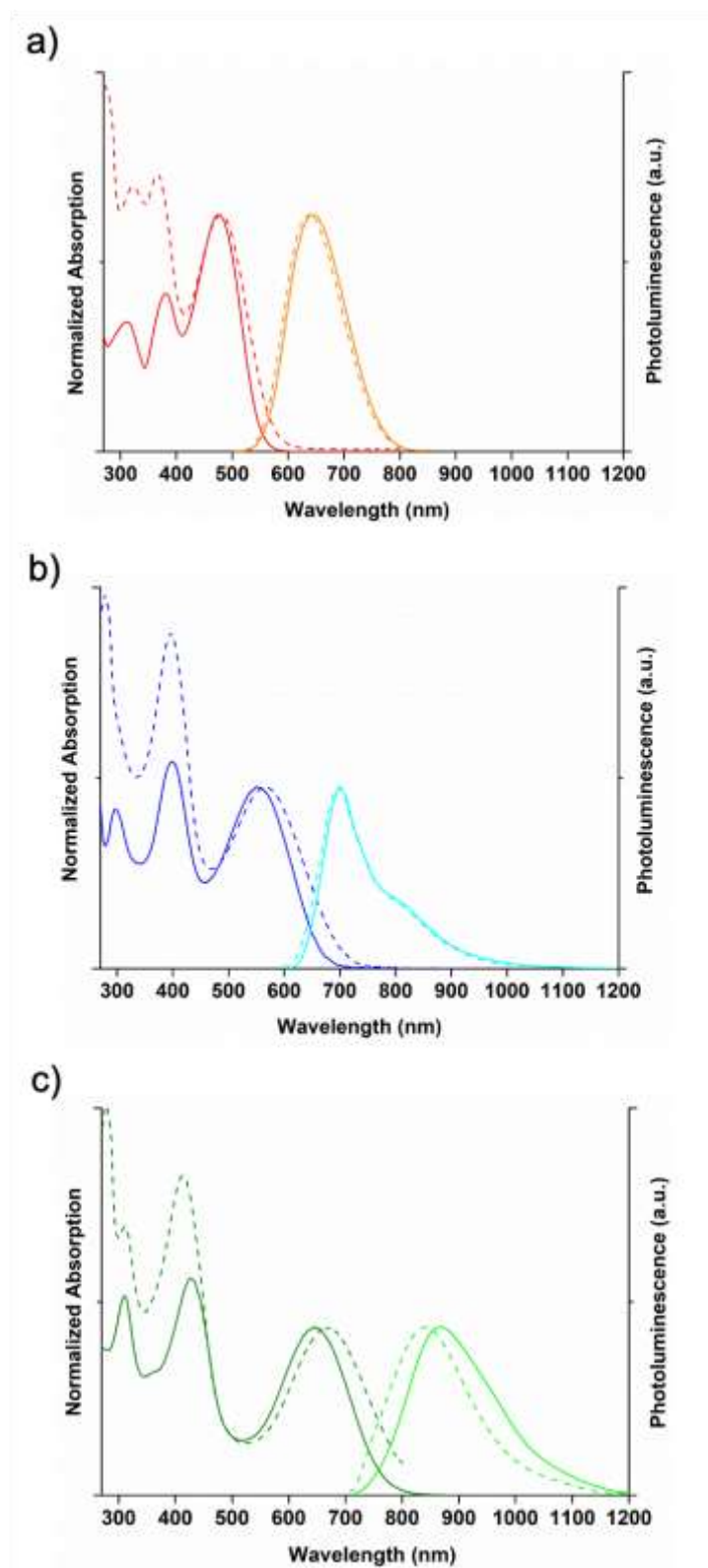


Figure S8. Absorption (left) and emission (right) spectra of a) **3a** (solid line) and **HSA-3a** (dashed line); b) **3b** (solid line) and **HSA-3b** (dashed line); c) **3c** (solid line) and **HSA-3c** (dashed line).

Table S2. Photophysical characterization of the compounds **3a**, **3b**, **3c** and **HSA-3a**, **HSA-3b**, **HSA-3c**

	$\lambda_{\text{Max}}^{\text{ABS}}$ (nm)	ϵ ($\text{M}^{-1}\cdot\text{cm}^{-1}$)	$\lambda_{\text{Max}}^{\text{Em}}$ (nm)	Stokes shift	ϕ_{Em}	τ_1 (ns)	τ_2 (ns)	χ^2 (ns)
3a	474	26875	644	170	0.5	6.08	-	1.09
HSA-3a	476	-	635	159	0.13	2.59	6.27	1.18
3b	550	14033	710	160	0.32	0.4	1.1	1.09
HSA-3b	565	-	706	141	0.14	0.6	1.6	1.08
3c	644	10200	868	224	0.00008	ND	ND	-
HSA-3c	671	-	840	169	0.00006	ND	ND	-

Electrophoresis

Native agarose gel electrophoresis was performed on an Owl Easycast B-Series Horizontal Gel Systems Model B2. Electrophoresis was performed in a 1 cm thick 5% agarose gel. Sodium carbonate buffer 100mM pH = 9 was used both as sample buffer and running buffer. 25 μg of protein conjugates, containing 10% v/v glycerol, were loaded into each well. HSA pre-stained with Coomassie Brilliant Blu was used as control. The run was performed applying a potential of 100V.



Figure S9. Native Agarose Gel Electrophoresis of HSA-oligothiophene bioconjugates. Lane 1. HSA stained by Coomassie Brilliant Blue; Lane 2, Non-stained **HSA-3a** bioconjugate; Lane 3, Non-stained **HSA-3b** bioconjugate; Lane 4, Non-stained **HSA-3c** bioconjugate.

Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) measurements have been performed using a Malvern Instruments DLS ZetaSizer Nano-ZS. DLS measurements were carried out on 2 μ M solutions of protein or bioconjugates in PBS 10mM pH 7.4 at room temperature.

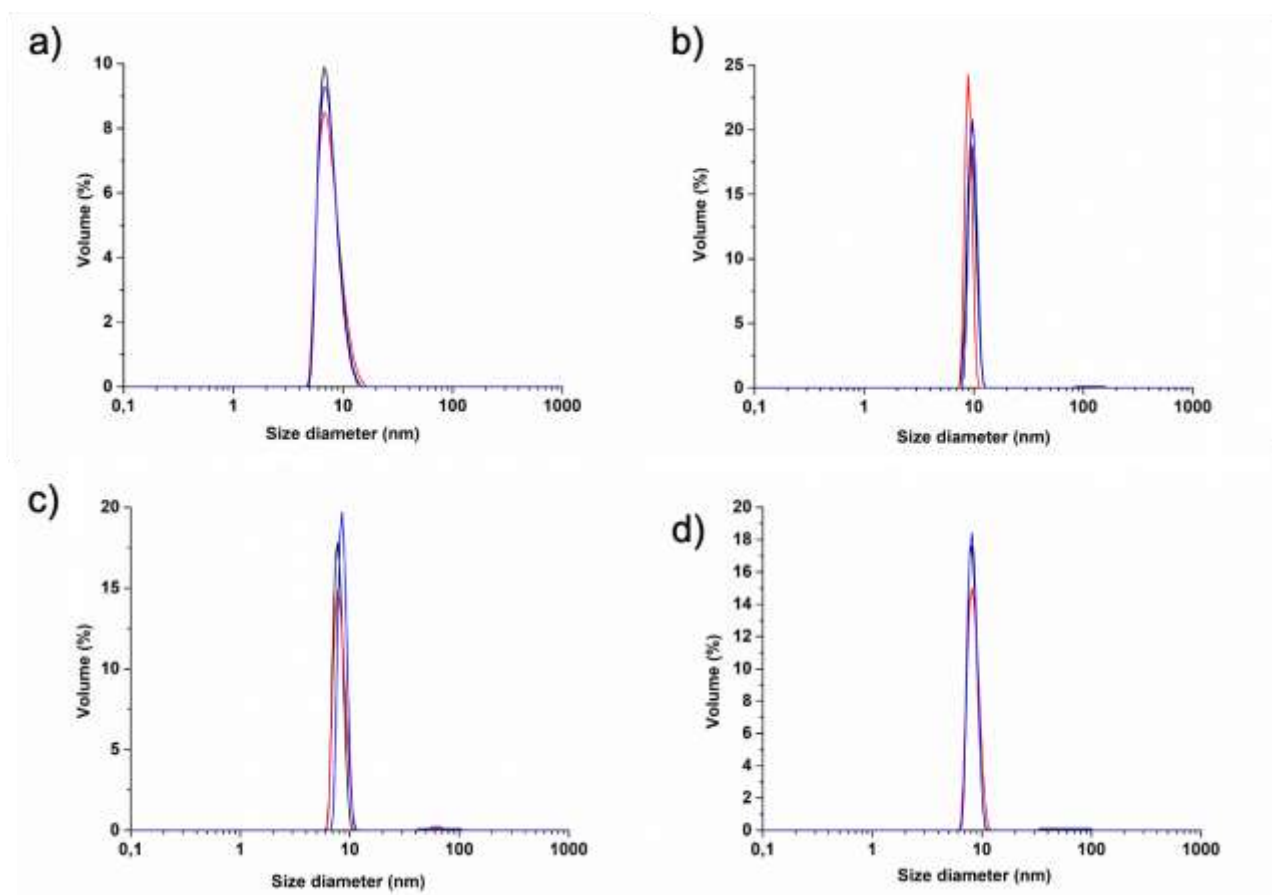


Figure S10. DLS distribution functions of a) HSA; b) **HSA-3a** bioconjugate; c) **HSA-3b** bioconjugate; d) **HSA-3c** bioconjugate. The measurements were repeated three times.

Circular Dichroism (CD)

CD spectra were collected using a spectropolarimeter JASCO J-715 under ambient conditions

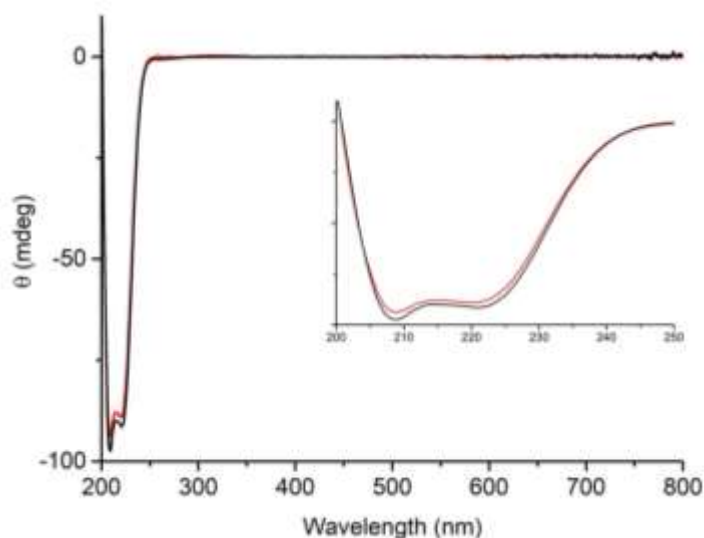


Fig S11. Circular dichroism (CD) spectra of **HSA** (black line) and **HSA-3a** bioconjugate (red line).

The inset shows a magnification of the 200-250 nm region.

We assessed the influence of oligothiophene binding on the structural changes of HSA through circular dichroism (CD) measurements. The HSA spectrum is characterized by two negative bands at 208 nm (π - π^*) and 220 nm (n - π^*) relative to the presence of α -helical content. As shown in Figure S11, the spectrum of the bioconjugate is superimposable to that of HSA. Indeed, the ratio of the CD intensity at 208 nm and 220 nm remains almost the same – indicating that the conformation of HSA is unchanged upon bioconjugation.

Amplex Red Peroxides Quantification

The ability to generate peroxides, upon irradiation with visible light, was evaluated using the Amplex Red assay. Colorless, nonfluorescent Amplex Red, reacts with peroxides to form colored, fluorescent resorufin, catalyzed by HRP. The concentration of the produced peroxides is calculated as the difference of resorufin generated by the irradiation of the photosensitizer and that of the references, i.e., iso-absorbing photosensitizer solutions, kept in the dark.

10 μl of Amplex Red 50 mM in DMSO was added to 1 ml of phosphate buffer 50 mM pH 7.4. Then 10 μl of HRP 0.4 mg/ml in PBS was added to the Amplex Red solution to obtain the final working solution. 90 μl of the solutions under investigation, containing different concentrations (0 μM 0.02 μM , 0.05 μM , 1 μM , 2 μM , 5 μM , 10 μM and 40 μM) of HSA-oligothiophenes in phosphate buffer 50 mM pH 7.4, were irradiated for 60 min with visible light (white LED Valex 30 W lamp at 30 cm distance from the cell-plate, irradiation power density on the cell plate = 2.4 mW/cm²; measured with the photo-radiometer Delta Ohm LP 471 RAD), on microtiter plates and 10 μl of Amplex Red working solution was added to each sample immediately after irradiation. After 30 min of incubation at room temperature, the fluorescence of the samples is read at 590 nm. A calibration curve generated using standard solutions of H₂O₂, was used to convert the fluorescence signal to the concentration of peroxides generated upon irradiation. Fluorescence measurements were carried using a Perkin Elmer EnSpire® Multimode Plate Reader.

ABMDMA Singlet Oxygen Assay

We used as ¹O₂ detector 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMDMA). The disodium salt of ABMDMA reacts with ¹O₂ to give an endoperoxide. This reaction is detected by the bleaching of ABMDMA. From the decline of the absorbance at 401 nm, the generation of ¹O₂ upon irradiation is determined. Iso-absorbing solutions at 555 nm of RB (15 μM) and HSA-oligothiophenes were prepared in deuterated PBS 10mM pH 7.4, with a concentration of the ABMDMA probe in the solutions of 25 μM . The solutions were stirred vigorously in dark conditions to ensure air saturation. The samples were irradiated at 555 nm and the bleaching of the absorption band of ABMDMA at 401 nm was monitored. The singlet oxygen quantum yield (Φ_{Δ}) was determined using Rose Bengal (RB) as the reference with a yield of 0.76 in PBS.¹ The Φ_{Δ} of HSA-3a was calculated by the following equation

$$\Phi_{\Delta}^S = \frac{k_S}{k_R} \times \Phi_{\Delta}^R$$

where k is the slope of the degradation rate of ABMDMA, S is the sample (**HSA-3a**), R is the reference (RB), and Φ_{Δ}^R is the Φ_{Δ} of the reference (RB).

Computational details

All computations were carried out using Gaussian16 suites of program.² The molecular structures of oligothiophenes **3a**, **3b** and **3c** were optimized using the DFT long-range corrected hybrid functional CAM-B3LYP and 6-31+G* basis set, a well-tested combination of DFT functional and basis-set, commonly used for the description of the electronic properties of this kind of molecules.³ The excited states were calculated using TD-DFT, at the same level of theory.³

References

- (1) Redmond, R. W.; Gamlin, J. N. A Compilation of Singlet Oxygen Yields from Biologically Relevant Molecules. *Photochem. Photobiol.* **1999**, *70*, 391-475.
- (2) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V. . M.; B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M. .; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, Jr., J. A. .; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J. .; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, N. J.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R. .; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G. .; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A.; D.; Farkas, O.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. Gaussian16, Wallingford, CT,. *Gaussian16 (Revision A.03)*. 2016.
- (3) Canola, S.; Mardegan, L.; Bergamini, G.; Villa, M.; Acocella, A.; Zangoli, M.; Ravotto, L.; Vinogradov, S. A.; Di Maria, F.; Ceroni, P.; et al. One- and Two-Photon Absorption Properties of Quadrupolar Thiophene-Based Dyes with Acceptors of Varying Strengths. *Photochem. Photobiol. Sci.* **2019**, *18*, 2180-2190.