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Fluorescent Probes

Azido-Substituted BODIPY Dyes for the Production of Fluorescent Carbon Nanotubes

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Abstract: A series of azido-dyes were synthesized through Knoevenagel reactions of an azido-BODIPY with aromatic aldehydes. The nature of the substituents allowed the fine tuning of their spectroscopic properties. The dyes were used to decorate oxidized multiwalled carbon nanotubes (ox-MWCNTs), bearing terminal triple bond groups, by CuAAC reactions, affording fluorescent materials. This decoration allowed the efficient determination of the internalization of the ox-MWCNT derivatives by different model cancer cells, such as MCF7.

Introduction

BODIPY-type molecules have found a widespread application as fluorescent probes.[1] The chemical stability of the BODIPY core goes along with its synthetic versatility.[2] The various reviews on the subject propose a large number of different structures.[3] Such structural variety allows the coverage of a great part of the UV/Vis spectrum, both in absorption and emission. However, despite their wide applications, BODIPY probes have been used sparingly with nanostructured carbonaceous materials.[4] In our ongoing study for the production of drug delivery systems based on carbon nanotubes (CNTs),[5] it was decided to decorate oxidized multiwalled carbon nanotubes (ox-MWCNTs), bearing terminal alkyne groups, with a fluorescent probe through a simple CuAAC reaction.[6] The choice for the use of MWCNTs is related, among other more practical reasons (cost, easy control of oxidation) to their being less addressed for use in biological systems. For this goal, it was necessary to synthesize simple dyes, bearing an azido group, the absorption and emission properties of which could be finely modulated to fulfill the requirements for the detection during biological tests. Herein, the synthesis of azido-substituted BODIPY dyes is described in which the reactivity of the methyl groups present on the pyrrole rings is exploited in a series of Knoevenagel reactions to extend the conjugation of the core and to obtain derivatives with spectroscopic properties that were modulated by the nature of the substituents. This synthetic approach is a straightforward way of extending the molecular conjugation of the BODIPY dye,[7] and is applied on azido-substituted BODIPY dyes for the first time. The availability of a library of BODIPY dyes bearing an azido group adds a useful alternative for their application in medicinal[8] and material chemistry[9] and, in our case, revealed to be a pratical method for the easy decoration of ox-MWCNTs. The coupling of properly modified ox-MWCNTs with BODIPY dyes afforded fluorescent material, and internalization of this material inside cells was monitored through cytofluorimetry and confocal microscopy analyses.

Results and Discussion

The starting material is represented by the BODIPY dye **5**, the synthesis of which has already been described.[10] Compound **5** is easily obtained from 2,4-dimethylpyrrole (**2**) and 4-nitrobenzaldehyde (**3**). Among the several different procedures that have been reported, we found more convenient to use the mechanochemical approach[11] up to the intermediate **3**, while the treatment with $BF_3{\cdot}Et_2O$ was performed in dry CH_2Cl_2.[10] The reduction of the nitro group with Fe powder/HCl afforded the corresponding amino group,[12] and the final conversion into the azido derivative **5** was performed with TMS-N_3 and isoamyl nitrite (Scheme 1).[13] The reactivity of the two methyl groups in positions 3 and 5 of the BODIPY core allowed to perform the Knoevenagel reaction with four different aromatic aldehydes, **6a–d**, to afford the corresponding mono- and disubstituted derivatives (Scheme 1). Previous examples of the Knoevenagel reaction on BODIPY derivatives were performed treating the dye with an excess of aromatic aldehyde in the pres-

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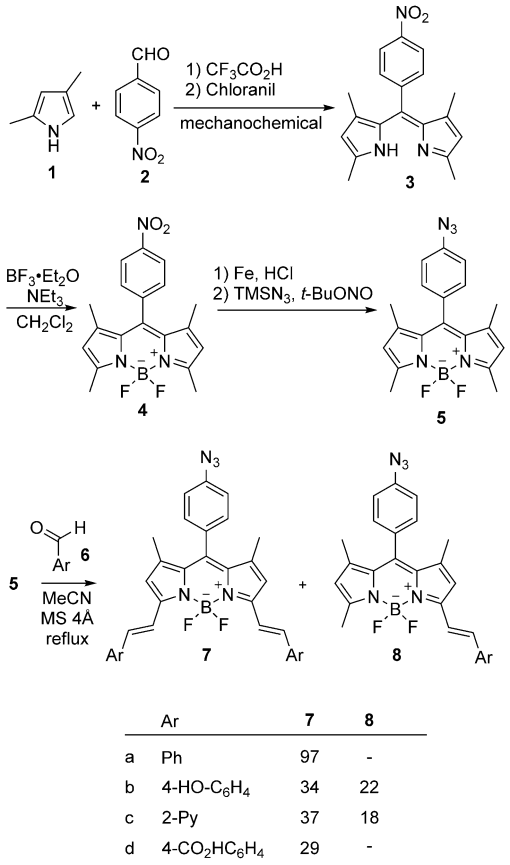


	Ar	7	8
a	Ph	97	-
b	4-HO-C_6H_4	34	22
c	2-Py	37	18
d	4-$CO_2HC_6H_4$	29	-

Scheme 1. Synthesis of compound **5** and its transformation into azido-substituted dyes **7a–d** and **8b,c**.

ence of acetic acid and pyrrolidine in refluxing toluene[14] or in absolute ethanol.[15]

However, the presence of the azido group on compound **5** made it sensitive to prolonged heating and required a compromise between conversion of the starting material and the final yields of the adducts. Even after a short reflux in toluene, the crude reaction material showed the presence of compounds in which the azido group had been lost. The best reaction conditions revealed to be reflux in MeCN for a very short time, checking the reaction mixture by TLC. The results are reported in Scheme 1. The reaction conditions were not optimized for the synthesis of the double or mono substituted derivatives but for the obtainment, when possible, of both compounds. This choice was due to the need to explore the potentiality of these compounds to cover a large part of the visible spectrum, in absorption as well as in emission. The reaction with benzaldehyde (**6a**) and 4-HO_2C-benzoic acid (**6d**) afforded exclusively the doubly substituted compound **7a**[13] and **7d**, respectively, while 4-hydroxybenzaldehyde (**6b**) and pyridine-2-carbaldehyde (**6c**) afforded a mixture of mono- and disubstituted compounds (Scheme 1). The structure of the final compounds were easily determined by ^{1}H NMR spectroscopy, showing the signals of the AX system related to the newly formed *E* double bonds.

In line with our expectation, the absorption spectra (Figure 1) covered a wide part of the visible spectrum ranging

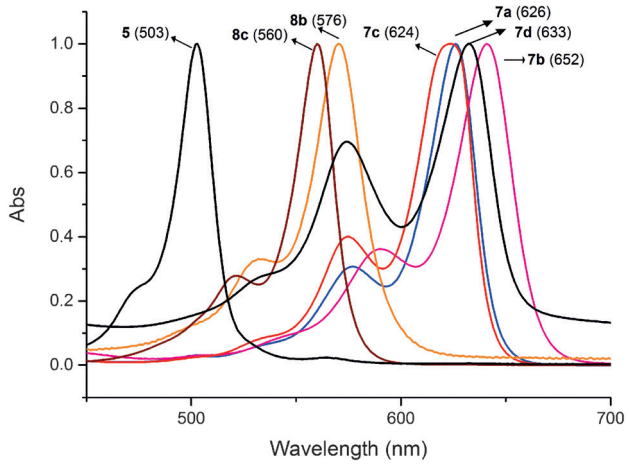


Figure 1. Absorption spectra (normalized) of compounds, **5**, **7b**, **8b**, **8c** (DMF), **7d** (H_2O/DMF), and **7a**, **7c** (CH_2Cl_2).

from 500 to 650 nm. For the sake of clarity, the UV spectra of the dyes were normalized. The molar attenuation coefficients of the compounds are reported.

Table 1 shows also a remarkable solvatochromic effect. Compound **7b**, sufficiently soluble in water, showed a 32 nm red-shift moving from CH_2Cl_2 to H_2O.

Also the maxima (see Figure 2) in the emission spectra were scattered, covering a range of 150 nm. The Stokes shift values are very low, as usually happens with this kind of fluorescent dyes, ranging from 8 nm (compound **8c**) to 18 nm (compound **7c**).[3]

The presence of the azido group on all the substituents allows their use in CuAAC reactions with compounds bearing a terminal triple bond. This reactivity was used for the decoration of ox-MWCNT **9** bearing terminal triple bonds (for the synthesis and characterization, see the Supporting Information, Figures S1–S7).[16,17] The CuAAC reaction with dyes **5**, **7b**, and **8c** was performed in DMF and afforded the fluorescent ox-MWCNT derivatives **10**–**12** (Scheme 2). The decoration of the MWCNTs afforded fluorescent materials for which absorption and emission properties can be easily tuned using differently substituted bodipy dyes. This material was found to be stable,

Table 1. UV max and molar attenuation coefficient ε of compounds **5**, **7**, and **8**.

Compound	UV_{max} [nm]	solvent	ε [L mol^{-1} cm^{-1}]
5	503	DMF	7.5×10^3
7a	626	CH_2Cl_2	1.4×10^5
	641	CH_2Cl_2	6.8×10^4
7b	652	DMF	6.5×10^4
	673	H_2O	8.7×10^3
8b	570	CH_2Cl_2	9.9×10^3
	576	DMF	8.7×10^3
7c	624	CH_2Cl_2	3.2×10^5
8c	560	CH_2Cl_2	2.0×10^5
	560	DMF	1.1×10^5
7d	633	H_2O/DMF	3.7×10^3

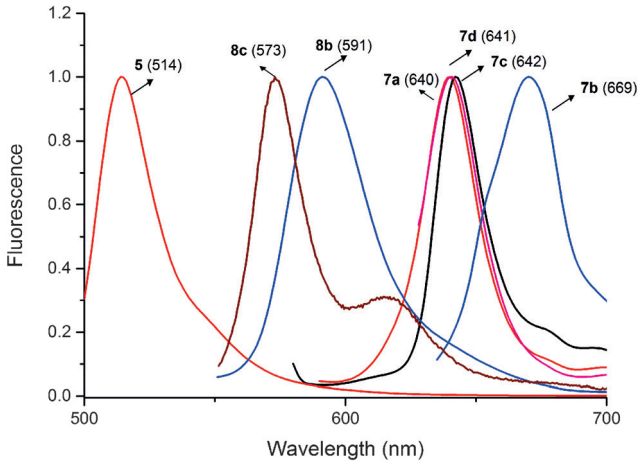


Figure 2. Emission spectra (normalized) of compounds **5**, **7b**, **8b**, **8c** (DMF), **7d** (H_2O/DMF), and **7a**, **7c** (CH_2Cl_2).

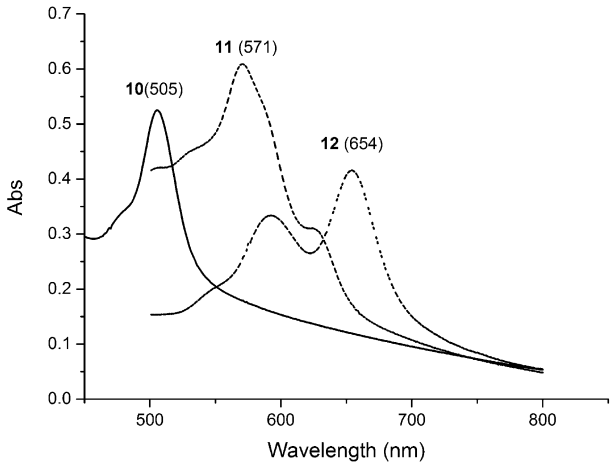
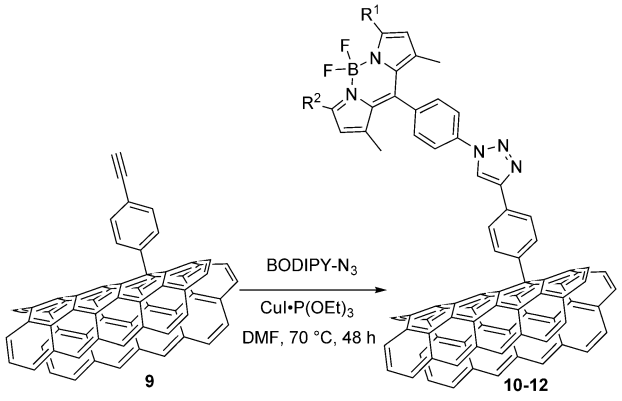


Figure 3. Absorption spectra of compounds **10**–**12**. All spectra were acquired in DMF (0.5 mg mL^{-1}).



BODIPY-N_3	R^1	R^2	product
5	Me	Me	**10**
8c	vinyl Py	H	**11**
7b	vinylC_6H_4OH	vinylC_6H_4OH	**12**

Scheme 2. The CuAAc reaction between CNT **9** and BODIPY dyes **5**, **7b**, and **8c**.

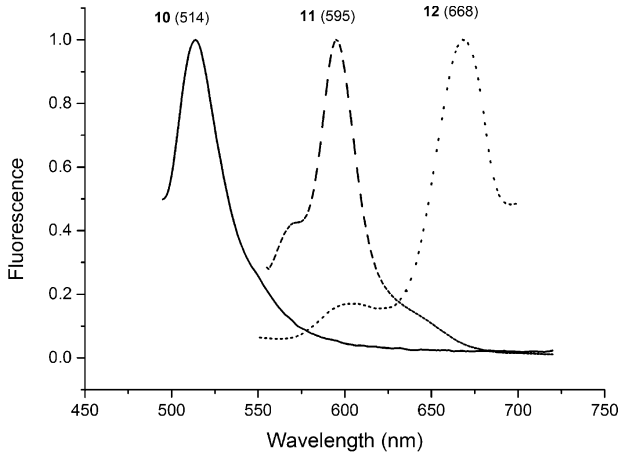


Figure 4. Emission spectra (normalized) of compounds **10**–**12**. All spectra were acquired in DMF (0.5 mg mL^{-1}).

as after several months, no apparent degradation of the fluorescence was observed.

Figures 3 and 4 show the absorption and emission spectra of compounds **10**–**12**. The decoration of the CNT walls did not alter significantly the absorption and the emission maxima of the BODIPY derivatives. This is evident when comparing the UV and fluorescence maxima of compounds **5** and **7b** with those of **10** and **12**, respectively: the wavelengths of absorption and emission (measured in DMF) are the same for each sample. This might be rationalized by considering that the fluorescent probe is efficiently solvated by DMF reducing to a minimum the interaction with the CNTs. On the contrary, compound **8c** shifted its UV and fluorescence maxima by 11 and 22 nm, respectively, when anchored to ox-MWCNTs to give compound **11**. This might be due to the presence of a pyridine ring on the dye and its interaction with the carboxylic groups present on the ox-MWCNTs.

A main issue, working with CNTs, is the determination of their functionalization degree obtained after a reaction. Very often this information is obtained through elemental or thermogravimetric analysis.

The presence of the BODIPY dyes, bearing a boron atom, allowed the use of inductively coupled plasma atomic emission spectroscopy (ICP-AES) for a quantitative determination of the functionalization degree of decorated ox-MWCNTs. In the case of compound **10**, the analysis revealed a presence of 12.4 mg of Boron per gram of material (1.14 mmol g^{-1} of material) while for compound **11** the analysis revealed the presence of 6.2 mg per gram of material (0.57 mmol g^{-1} of material). The confirmation of the capacity of the fluorescent probes to show the internalization of CNTs inside cancer cells was achieved by a series of cytofluorimetric analyses performed on three different cancer cell lines : MCF7 (human breast adenocarcinoma), PC3 (prostatic small cell carcinoma), and HT29 (human colon cancer) after incubation with compound **10**, **11**, and **12** (Figure 4 and Table 2).

Table 2. Fluorescence data from cytofluorimetry analysis after incubation of three different cell lines with compounds **10**–**12**.[a]

	10		11		12	
Cell line	Control	Treated	Control	Treated	Control	Treated
HT29	136	597	344	4190	69	1350
PC3	93	531	247	4879	158	1260
MCF7	180	2650	275	1380	275	2900

[a] Each cell culture was incubated for 90 min with a water dispersion of the decorated nanotubes **10**–**11** (10 μg mL^{-1}). Data reported in the table represent the mean values of the fluorescence of the cells.

Figure 5 shows the results of three cytofluorimetric analyses performed with compounds **10**–**12** on MCF7 cancer cell lines. The Figure shows the remarkable increase of fluorescence of the cells, which is directly related to the internalization of the CNTs. Table 2 summarizes all of the cytofluorimetric data collected for the three different cancer cell lines. In all cases there is a net increase of the cellular fluorescence, demonstrating that the decorated carbon nanotubes are efficiently internalized by different cell lines.[18] While it is not possible to compare the internalization degree of the different compounds inside one kind of cell cultures, as any enhancement or quenching effect might be different, it is possible to compare the difference between the internalization degrees of the same compound inside different kind of cells. For example, MCF7 cells showed a much higher fluorescence when incubated with **10** respect to HT29 and PC3 (five times the final fluorescence value). The opposite holds for **12** for which HT29 and PC3 cells showed a threefold value of the final fluorescence. Since the functionalization degree of the compound used is the same, it is possible to correlate, at least qualitatively, the degree of internalization with the enhancement of fluorescence.

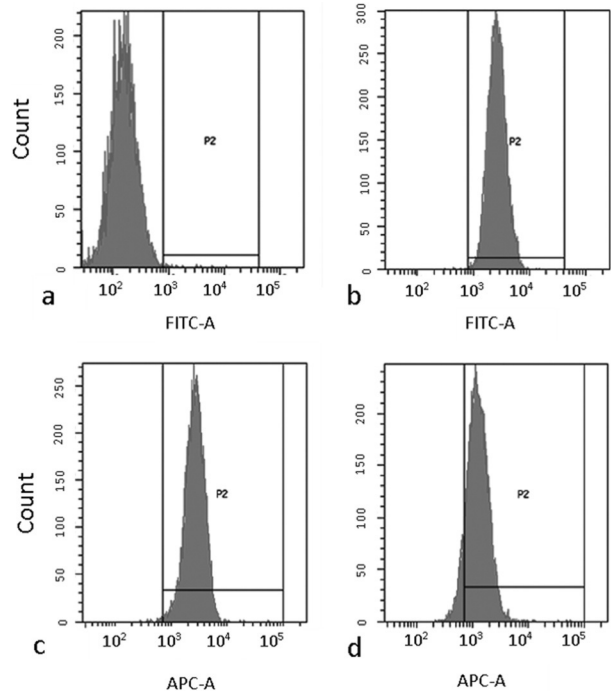


Figure 5. Cytofluorimetric analysis of MCF7 cell lines after incubation with fluorescent CNTs: a) control experiment, b) incubation with **10**, c) incubation with **11**, d) incubation with **12**.

Finally, compound **11** was used in a confocal microscopy analysis performed on MCF7 cells after incubation (Figure 6).

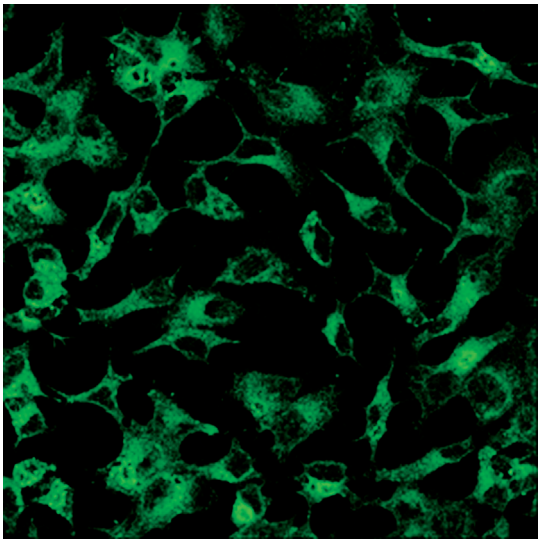


Figure 6. Confocal microscopy analysis of a MCF7 cell culture after incubation with MWCNT **11**.

Compound **11** was chosen since it best fitted the excitation frequency (571 nm) of the laser used in the confocal microscopy analysis. Despite the fact that MCF7 cells were those that evidenced the smaller increase in fluorescence upon incubation with compound **11**, the confocal analysis afforded a clear picture (Figure 6): the fluorescence of compound **11** is well distributed in the cytoplasm of the cells while the nuclei remain unaffected. Concerning the amount of material internalized, during the biological tests it was not possible to determine any difference between the cell cultures incubated with simple Ox-MWCNTs and those incubated with compound **11**.

To verify any possible toxic effect of the nanostructured materials, MTT assays were performed and the results are given in the Supporting Information, Figure S8: neither simple ox-MWCNTs nor compound **11** show any sign of cytotoxicity towards MCF7 cell lines using a concentration of 10 mg mL^{-1} of the material.

Conclusion

A series of Knoevenagel reactions of an azido-bodipy derivative allowed the production of a small library of azido-substituted fluorescent dyes, the spectroscopic properties of which are tuned by the nature of their substituents. The presence of an azido group on the fluorescent dyes allowed their easy anchoring onto properly substituted ox-MWCNTs. This affords fluorescently labeled nanostructured carbon materials that can be internalized inside cells after a brief incubation time. Fur-

ther studies for use of these materials as platform for the selective delivery of drugs into cancer cells are ongoing in our laboratories.

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Keywords: BODIPY · carbon nanotubes · click reaction · fluorescent probes

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