Bioorganic & Medicinal Chemistry Letters 20 (2010) 6001–6007

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

On the use of 2,1,3-benzothiadiazole derivatives as selective live cell fluorescence imaging probes

Felipe F. D. Oliveira [a,b], Diego C. B. D. Santos [a,b], Alexandre A. M. Lapis [c], José R. Corrêa [a,b], Alexandre F. Gomes [d], Fabio C. Gozzo [d], Paulo F. Moreira Jr. [e], Virgínia C. de Oliveira [a,b], Frank H. Quina [b,e], Brenno A. D. Neto [a,b,*]

[a] Laboratory of Medicinal and Technological Chemistry, University of Brasília (IQ-UnB), Campus Universitário Darcy Ribeiro CEP 70904-970, PO Box 4478, Brasília, DF, Brazil
[b] National Institute of Catalysis (INCT-Catalysis), Brazil
[c] Universidade Federal do Pampa, Unipampa/Urcamp, Bagé, RS, Brazil
[d] Institute of Chemistry, State University of Campinas, Brazil
[e] Institute of Chemistry, USP, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 6 August 2010
Revised 12 August 2010
Accepted 16 August 2010
Available online 20 August 2010

Keywords:
Synthesis
Benzothiadiazole
ESIPT
Live cell-imaging
Stem-cells

ABSTRACT

Newly designed 2,1,3-benzothiadiazole-containing fluorescent probes with four excited state intramolecular proton transfer (ESIPT) sites were successfully tested in live cell-imaging assays using a confluent monolayer of human stem-cells (tissue). All tested dyes were compared with the commercially available DAPI and gave far better results.

The development of new and efficient fluorophores to detect, quantify or to observe cellular process is currently of a great interest and has been recently reviewed.[1] The design and synthesis of new fluorophores that display any induced fluorescence signal change (e.g., intensity and/or emission wavelength or shape) when a fluorophore-containing probe specifically binds to a target is particularly desirable.[2] Ultrafast excited state intramolecular proton transfer (ESIPT) processes greatly increase the stability of a molecule in the excited state, avoiding undesirable photochemical reactions.[3] Additionally, it has been demonstrated that the ESIPT process is microenvironmentally sensitive; thus, when the emission is characteristics of an ESIPT compound is dependent on its microenvironment, its fluorescence emission are expected to change under different conditions.[4] Indeed, molecules showing ESIPT fluorescence have been employed as probes of microenvironments in biological systems.[5] Molecules that contain two sites capable of undergoing ESIPT processes, a molecular architecture that greatly increases the molecule's stability in the excited state, have also been reported.[6]

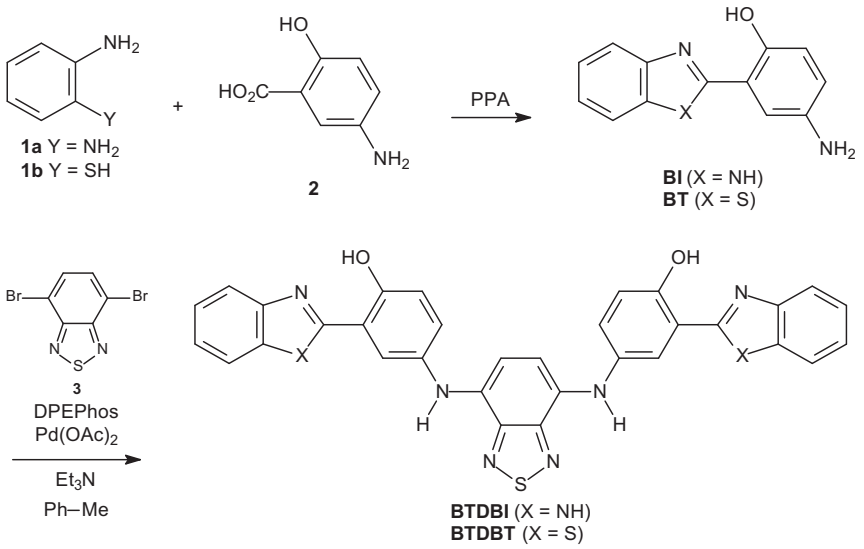
In previous works, we showed that the 2,1,3-benzothiadiazole (BTD)[7] core and its derivatives are potential candidates for use in organoelectronic devices,[8] as molecular fluorescent probes for selective dsDNA detection[9a] and that they are efficiently used in real-time PCR.[9b] In the present work, we designed and synthesized new 2,1,3-benzothiadiazole derivatives having four sites potentially capable of participating in ESIPT processes and report photophysical and electrochemical data and preliminary studies of application in live cell-imaging in a selective staining of dsDNA in human stem-cells.

The dyes were synthesized in good yields as shown in Scheme 1 and were fully characterized (see Supplementary data). The known ESIPT fluorophores **BI** and **BT** were synthesized using a previously described methodology by Campo et al.[10] The new derivatives, **BTDBI** and **BTDBT**, were synthesized by Buchwald-Hartwig amination of 4,7-dibromo-2,1,3-benzothiadiazole (**3**), employing an adaptation of a procedure recently described by Hirao and coworkers.[11]

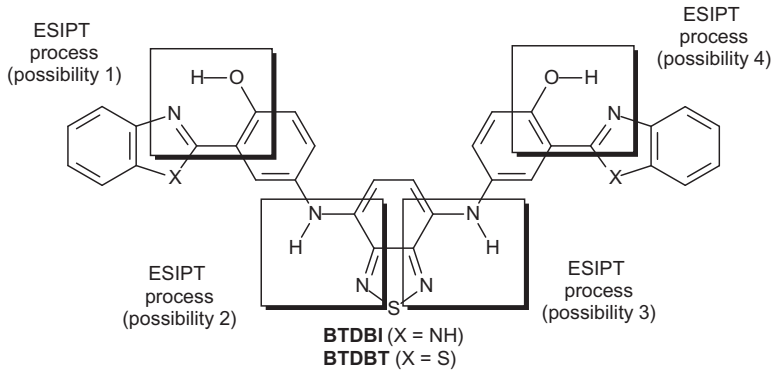
In the Supplementary data, we describe the general ESIPT process (see Scheme S1). As indicated in Scheme 2, the molecular architecture of the dyes **BTDBI** and **BTDBT** has been designed to permit, in principle, any one of four different ESIPT processes. In two of the four possible ESIPT processes, intramolecular proton

* Corresponding author. Tel.: +55 61 31073867; fax: +55 61 32734149.
E-mail address: brenno.ipi@gmail.com (B.A.D. Neto).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.08.073



Scheme 1. Synthesis of the dyes **BI**, **BT**, **BTDBI** and **BTDBT**.



Scheme 2. Four possibilities for ESIPT process to undergo in the excited state of the fluorophores **BTDBI** and **BTDBT**.

transfer would occur via a six-membered ring (possibilities 1 and 4) and, in the other two, via a five-membered ring (possibilities 2 and 3). Both five- and six-membered ring ESIPT processes have been studied in other systems, where they efficiently stabilize the molecules in the excited state.[5,12] Moreover, upon intercalation, one side of the symmetrical dye would be able to allow the molecule to undergo ESIPT process in two different sites.

Differential scanning calorimetry (DSC) was used to determine the thermal properties of all four compounds (melting or decomposition temperatures). Photophysical properties of all compounds were also investigated and are summarized in Table 1.

The large observed Stokes shift is a consequence of the high stability in the excited state and the ESIPT process. Moreover, such a large Stokes shift and solvent-dependent emission indicates effective intramolecular charge transfer (ICT) in the excited state between the **BTD** unit and the substituent at positions 4 and 7.[14] It is well known that molecules that undergo ESIPT processes display a large Stoke shift. **BI**, **BT**, **BTDBI** and **BTDBT** exhibit large molar extinction coefficients (ε) in acetonitrile ($\log \varepsilon = 3.90$, 3.72, 3.91 and 3.93, respectively, for 5.00×10^{-5} M solutions). **BI** and **BT** are almost insoluble in water, but the use of ultrasound permits the preparation of highly dilute solutions for which no precipitation is noted after standing for a few minutes. The derivatives **BTDBI** and **BTDBT** showed higher solubility in water. The strategy of combining the advantages of a **BTD**[15] core with ESIPT processes thus appears to be a good approach for improving the photophysical properties of the new compounds.

Spectrophotometric titrations: All dyes were assayed by spectrophotometric titration against dsDNA (calf thymus) or the BSA protein (b*ovine serum albumin*). Water (M*illi-Q purified*) was used as

Table 1
UV-vis and fluorescence data for **BI**, **BT**, **BTDBI** and **BTDBT**

Compd	λ_{max} (abs)[a]	λ_{max} (em)[a]	Stokes shift[a]	Φ_f
BI	346 (344),[b] 361 (362),[b] 338	478, (479),[b] 425, (423),[b] 456	132, 64, 127	0.022
BT	371 (369),[b] 389 (389),[b] 359	513, (508),[b] 478, (477),[b] 462	142, 89, 103	0.0046
BTDBI	348, 356, 353	444, 438, 462	96, 82, 109	0.016
BTDBT	344, 345, 345	445, 440, 463	101, 95, 118	0.016

[a] EtOH, MeCN, H_2O, respectively.
[b] Literature.[13]

medium for these experiments as a prelude to future cellular experiments (live cell-imaging). The presence of phosphate buffer (pH 7.0) did not significantly alter the results obtained. Supposedly, the binding with the biomolecule would result in a different spectrum during the titration. The use of very dilute solutions of the dyes (1.0×10^{-5} M) failed to reveal significant changes in the spectra (see Figs. S1 and S2 in the Supplementary data). However, with solutions of a much higher concentration (50 μM),[16] better results were obtained. Figure 1 shows representative results for the dye **BTDBT**. Results for all of the fluorophores are provided in the Supplementary data (Figs. S3–S6).

The use of benzoxazole,[17a] benzothiazole[17b] and benzoimidazole[17c] derivatives for protein detection has been described, so spectral changes for our compounds could be expected in the presence of BSA protein. Indeed, spectrophotometric titrations gave better results for protein detection than for the nucleic acid under the conditions studied.

Spectrofluorimetric titrations: Spectrofluorimetric detection is far better when compared to spectrophotometric detection. Indeed, spectrofluorimetric titrations gave very interesting results even at low dye concentrations. The best results for protein and nucleic acid detection were obtained using **BTDBT** (Fig. 2; see Figs. S7–S10 in the Supplementary data for the data for all dyes). However, for protein detection **BTDBT** was not as good as expected.

In contrast, **BTDBI** gave rather poor results (Fig. S9 in the Supplementary data), most probably due to its high hydrophilicity. This lowers the affinity of the dye for the biomolecules and results in only small spectral changes, making it less than desirable as a probe for the spectrofluorimetric detection and quantification of biomolecules. Although **BI** and **BT** gave reasonable results, their poor solubility means that they are not very adequate for detecting biomolecules. Based on these considerations, **BTDBT** (Fig. 2) is clearly the best fluorophore among the four tested in this work.

pH dependence of the fluorescence: The pH dependence of ESIPT processes is well known and the species involved can be neutral, anionic or cationic, depending on the pH range.[18] For instance, we have recently shown this pH dependence for the substrate of IGPS enzyme from *Mycobacterium tuberculosis* bacillus and its importance in tryptophan biosynthesis.[19] In this work, very different behaviors were observed for the four dyes (Fig. 3). While the fluorescence spectra of **BI** and **BT** are strongly dependent on the solution pH (shape, intensity and absorption maximum shift), only the fluorescence intensity of **BTDBI** and **BTDBT** changes, with the shape of the spectra being almost unchanged. While **BI** and **BT** are strong dependent on the pH value (shape, intensity and absorption maximum shift), the large π-extension of **BTDBI** and **BTDBT** makes the shape of the spectra almost unchanged. The intensity changes, as expected, but the intrinsic fluorescence remains unchanged due to the large π-extension. Moreover, the large extension of the π-conjugation turns both, **BTDBI** and **BTDBT**, highly stable. Despite the pH value, that could lead both BTD-derivatives to anionic or cationic species, almost no absorption maximum shift is noted. It is due to the fact that the charge (positive or negative) is very well stabilized in the highly extended π-systems. A reference work has been published[20] demonstrating that in aqueous solutions at near neutral pH values species have a tendency to be predominantly neutral, whereas at lower and higher pH values they tend to be cationic and anionic, respectively. Nevertheless, the fact that **BTDBI** and **BTDBT** have the highest fluorescence in the range of physiological pH values makes these fluorophores excellent candidates for biological applications such as live cell-imaging.

It is noted that for **BTDBI** and **BTDBT**, the pH-induced changes in fluorescence intensity are reversible, indicating that protonation and deprotonation are reversible and no decomposition is noted. This was not the case for **BI** and **BT**, which probably suffer some degradation in very acidic or basic solutions.

Electrochemical analysis: Since protein detection was not very effective, and the selectivity was very good for dsDNA, we decided to limit our study of electrochemical methods to DNA detection. DNA interactions were studied by using cyclic voltammetric analysis. The cyclic voltammograms (CV) are shown in Figure 4.

Larger electrochemical windows were not tested in order to avoid any solvent oxidation and/or reduction. In all cases, we observed multielectron irreversible reduction steps during cathodic potential sweeps in the presence of DNA or for pure DNA itself. No cathodic reduction processes were observed for any of the pure fluorophores in the same electrochemical window. However, the anodic sweep for the dyes **BI** and **BT** provided very interesting results. **BI** showed an anodic peak at 0.48 V associated with the peak at 0.38 V in a reversible charge transfer process. Upon DNA addition, a shift of these peaks to 0.65 and 0.27 V is observed and the charge transfer is quasi-reversible. **BT** also showed an anodic peak at 0.70 V associated with the peak at 0.53 V in a reversible charge transfer process. When the DNA is added to the system, the peak at 0.70 V is shifted to 0.57 V and the oxidation process become irreversible, since no peak is noted during the reverse sweep. In contrast, neither **BTDBI** nor **BTDBT** showed any oxidation or reduction processes in the electrochemical window studied, clearly indicating their high electrochemical stability. Upon DNA addition, there was a pronounced change in the current (μA), especially for **BTDBT**, consistent with its high affinity for dsDNA. In some recently published works,[21] a similar behavior could be noted upon dsDNA addition to a small molecule-containing solution. The substantial change in the peak current can be attributed to the

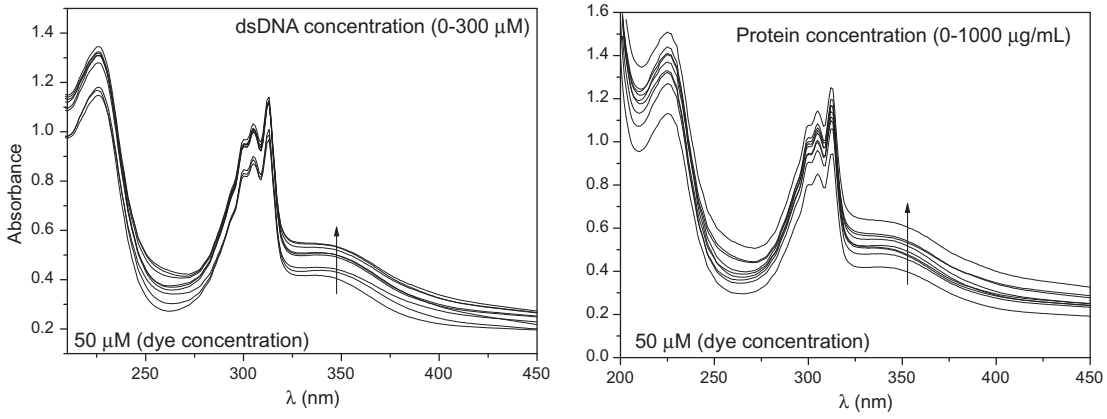


Figure 1. Typical spectral behavior for the spectrophotometric titration of BTDBT with dsDNA (left) or BSA (right).

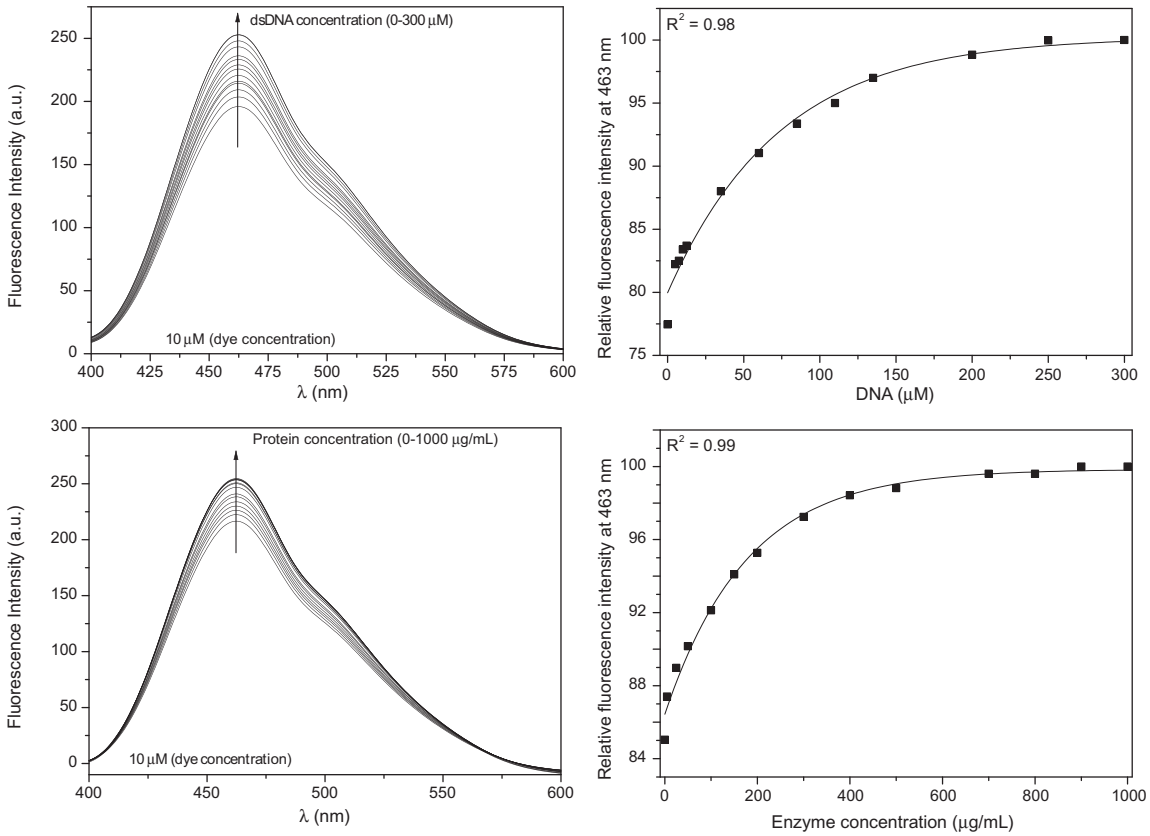


Figure 2. Representative spectrofluorimetric titration of **BTDBT** with dsDNA (upper) or BSA (lower).

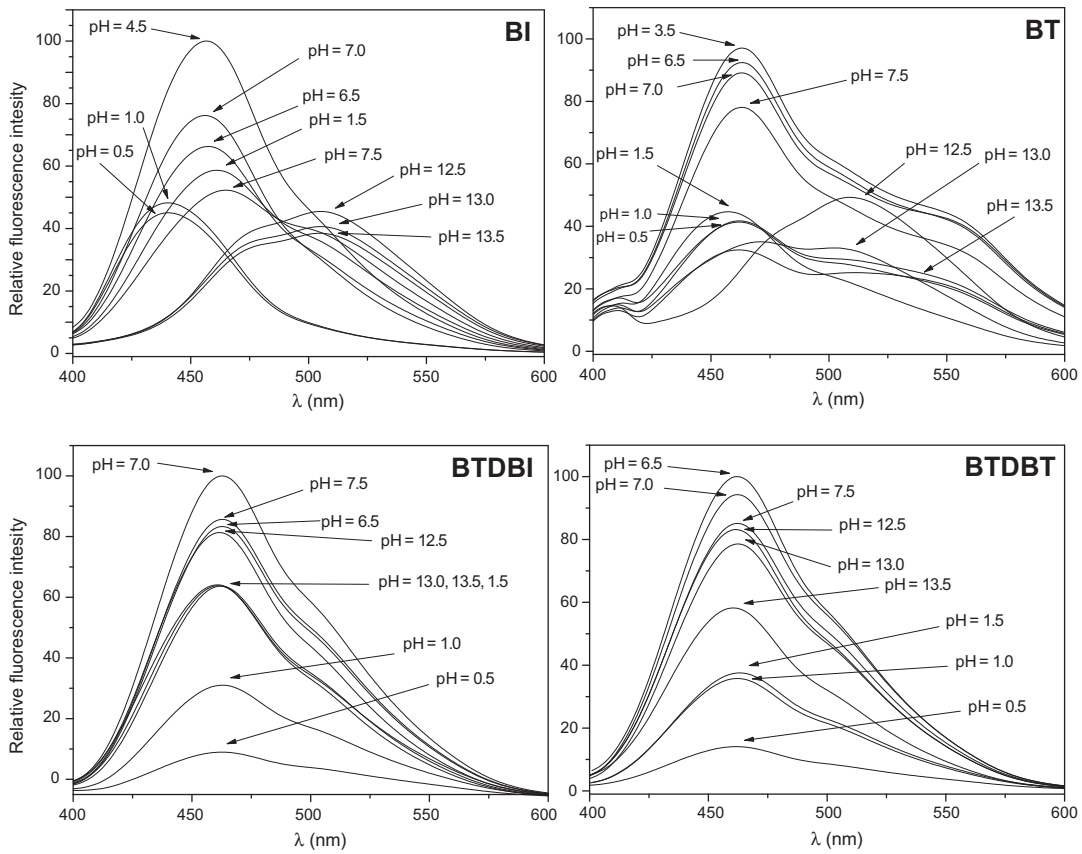


Figure 3. Dependence of the probe fluorescence on pH in aqueous solutions. See Supplementary data (Figs. S11–S12) for the spectra at all of the pH values tested for the four dyes.

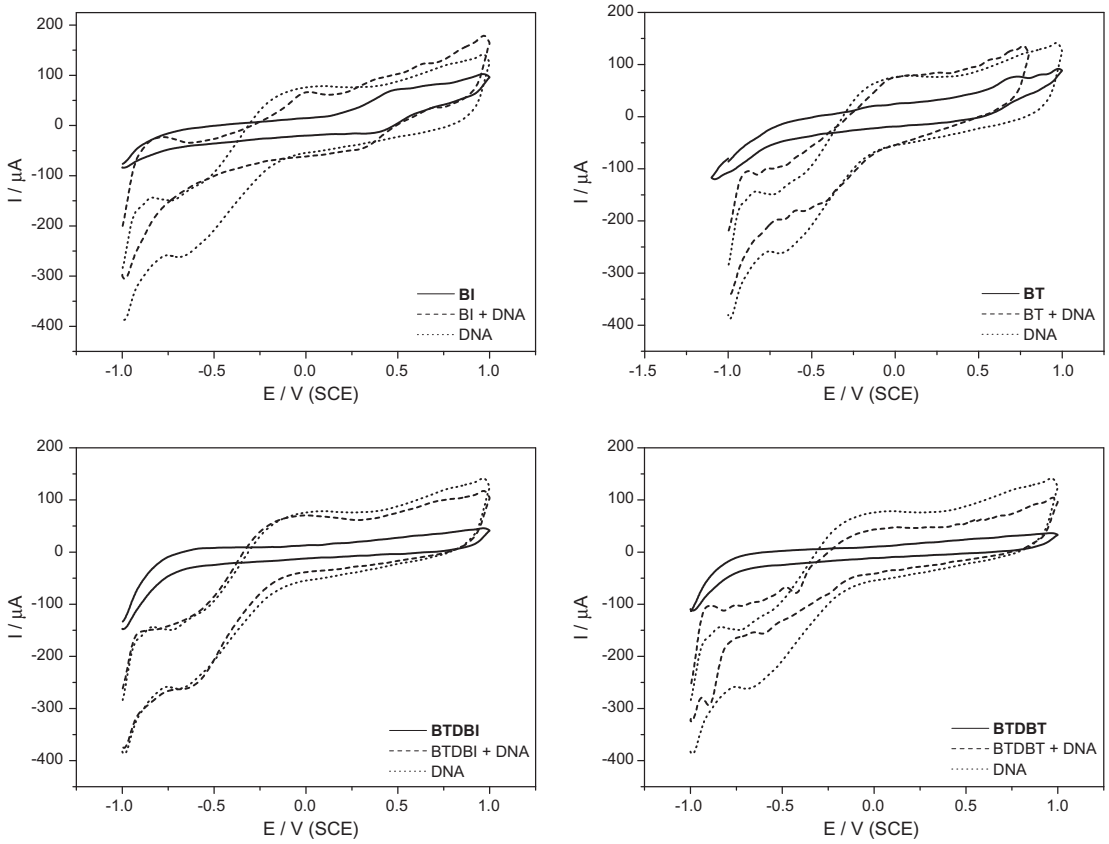


Figure 4. Cyclic voltamograms for all four dyes (1.00 mM). In MeCN (0.10 M $TBAPF_6$) recorded at a scan rate of 300 mV/s. The dsDNA concentration was 300 μM in all cases. Note the large difference in the current (μA) for **BTDBT** between the biomolecule (DNA) alone and for DNA + **BTDBT**.

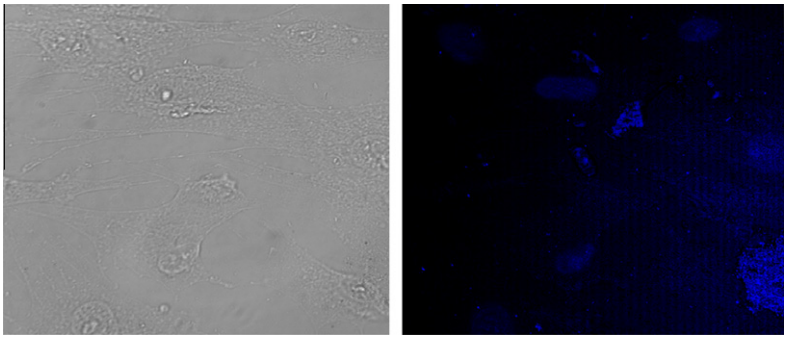


Figure 5. Live cell-imaging using **DAPI** (commercially available) and adhered human stem-cells. Note in this picture that **DAPI** is not as selective as the designed probes and is also visualized in the cellular membrane and in other cellular components (right picture). Phase contrast (left) and fluorescence profile (right).

decrease in free fluorophore concentration due to the formation of a diffusing, heavy molecular weight dye-DNA adduct. We recently showed the high affinity of BTD-containing intercalators and dsDNA.[9b] Peak potential shift could be attributed to the intercalation of a planar and rigid moiety of the molecule into the stacked base pairs domain of dsDNA, as discussed elsewhere.[22]

Live cell-imaging analysis: Live cell-imaging experiments were performed using human stem-cells and murine macrophage cells (see Experimental details in the Supplementary data). In these experiments, all dyes were tested with both suspended and adhered cells. Initially, suspended cells (macrophage cells) were used for testing the viability of the use of the dyes. The results showed that dsDNA was preferentially stained in all cases (see Figs. S13–S16 in the Supplementary data). Moreover, a more complex cellular model consisting of a confluent monolayer of human stem-cells (tissue) was then tested. All four dyes were capable of transposing the cellular membrane and successfully and selectively stained dsDNA inside the human stem-cells (see Fig. 5 and Figs. S17–S21 of the Supplementary data for full-size images).

It is important to highlight the importance of a selective dsDNA staining. The high selectivity allows the study of any nucleic acid process without interfering in any enzyme or protein related to a specific process.

Despite the potentially quite useable staining result, **BI** and **BT** precipitated during the experiment due to their very low solubility in aqueous media, making them less than appropriate for this kind of experiment, especially because they can precipitate in the intracellular media.

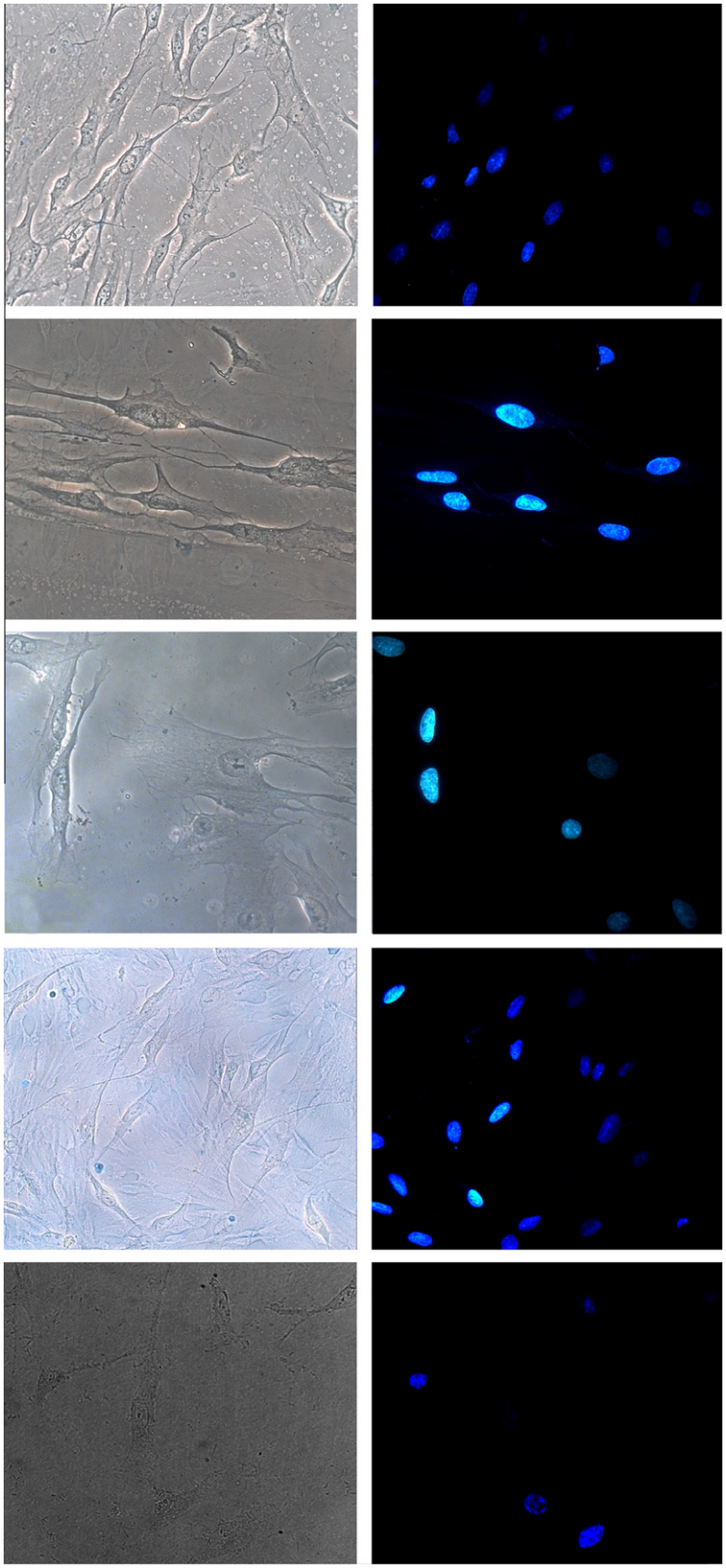


Figure 6. Live cell-imaging of adhered human stem-cells using all four dyes and **DAPI** (commercially available). Phase contrast (left) and fluorescence micrographs (right). From top to bottom, the dyes are: **BI**, **BT**, **BTDBI**, **BTDBT** and **DAPI**. Note that **BTDBT** gave the most efficient dsDNA staining.

The designed fluorophores **BTDBI** and **BTDBT**, however, proved to be exceptional for this purpose, due in large part to their high dsDNA affinity and their chemical and photochemical stability, mainly **BTDBT**. The cellular experiments leave no doubt about the high affinity of the new dyes for dsDNA. The commercially available dye **DAPI**, which is among the most widely used molecular probe for dsDNA staining, was tested in the same confluent monolayer of human stem-cells in order to compare the results with those for our molecular probes. It is known that **DAPI** commonly associates with other cellular components in addition to dsDNA, since its selectivity is not as good as expected to be. For instance, **DAPI** adsorbs to the cellular membrane resulting in a strong background signal even at relatively high dilutions (Fig. 5 and Fig. S22).

In the case of both **BTDBI** and **BTDBT** (Fig. 6), the images are quite clear, with little or no background, indicating that no significant amount of these two fluorophores may remain adsorbed in the membrane. Actually, they are capable of transposing the membrane without adhering to it. Although both designed dyes are much better than **DAPI**, comparison of the results for **BTDBI** and **BTDBT** indicates that **BTDBT** gave the best overall results as a live-cell dsDNA stain.

In summary, we have demonstrated that the molecular architecture of the new dyes **BTDBI** and **BTDBT**, with four regions that can potentially participate in ESIPT processes, provides extremely efficient stabilization of these molecules in the excited state. The fluorescence of **BI** and **BT** is strongly pH-dependent and these dyes decompose at very acid or very basic pH. The newly designed fluorophores, **BTDBI** and **BTDBT**, were very pH-stable, and it was shown that the shape of their fluorescence spectra is independent of the solution pH and their fluorescence emission intensities are highest around physiological pH. In live cell-imaging experiments, **BTDBT** gave better results for staining of dsDNA than either **BTDBI** or **DAPI**, while **BI** and **BT** precipitated during the experiment. The use of **BTDBT** to accompany molecular processes in human stem-cells are underway in our laboratories and will be published in due course.

Acknowledgments

CAPES, CNPq, FINEP, Finatec, FAPESP and FAPDF are acknowledged for partial financial support. BAD Neto is in debt with Prof. Sônia M. Freitas and Prof. Paulo A.Z. Suares. B.A.D. Neto, F.C. Gozzo and F.H. Quina also thank CNPq for research fellowships.

Supplementary data

Supplementary data (spectral data) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.073.

References and notes

1. (a) Nolan, E. M.; Lippard, S. J. *Acc. Chem. Res.* **2009**, *42*, 193; (b) Neto, B. A. D.; Lapis, A. A. M. *Molecules* **2009**, *14*, 1725.
2. (a) Cheng, T. Y.; Xu, Y. F.; Zhang, S. Y.; Zhu, W. P.; Qian, X. H.; Duan, L. P. *J. Am. Chem. Soc.* **2008**, *130*, 16160; (b) Feng, S. H.; Kim, Y. K.; Yang, S. Q.; Chang, Y. T. *Chem. Commun.* **2010**, *46*, 436; (c) Martin, R. M.; Leonhardt, H.; Cardoso, M. C. *Cytometry A* **2005**, *67A*, 45.
3. Sun, W. H.; Li, S. Y.; Hu, R.; Qian, Y.; Wang, S. Q.; Yang, G. Q. *J. Phys. Chem. A* **2009**, *113*, 5888.
4. Ohshima, A.; Momotake, A.; Arai, T. *J. Photochem. Photobiol., A* **2004**, *162*, 473.
5. Dennison, S. M.; Guharay, J.; Sengupta, P. K. *Spectrochim. Acta, Part A* **1999**, *55*, 1127.
6. Borowicz, P.; Grabowska, A.; Les, A.; Kaczmarek, L.; Zagrodzki, B. *Chem. Phys. Lett.* **1998**, *291*, 351.
7. Neto, B. A. D.; Lopes, A. S.; Wust, M.; Costa, V. E. U.; Ebeling, G.; Dupont, J. *Tetrahedron Lett.* **2005**, *46*, 6843.
8. (a) Mancilha, F. S.; Neto, B. A. D.; Lopes, A. S.; Moreira, P. F.; Quina, F. H.; Goncalves, R. S.; Dupont, J. *Eur. J. Org. Chem.* **2006**, 4924; (b) Neto, B. A. D.; Lopes, A. S. A.; Ebeling, G.; Goncalves, R. S.; Costa, V. E. U.; Quina, F. H.; Dupont, J. *Tetrahedron* **2005**, *61*, 10975.
9. (a) Neto, B. A. D.; Lapis, A. A. M.; Mancilha, F. S.; Vasconcelos, I. B.; Thum, C.; Basso, L. A.; Santos, D. S.; Dupont, J. *Org. Lett.* **2007**, *9*, 4001; (b) Neto, B. A. D.; Lapis, A. A. M.; Mancilha, F. S.; Batista, E. L., Jr.; Netz, P. A.; Rominger, F.; Basso, L. A.; Santos, D. S.; Dupont, J. *Mol. BioSyst.* **2010**, *6*, 967.
10. Campo, L. F.; Correa, D. S.; de Araujo, M. A.; Stefani, V. *Macromol. Rapid Commun.* **2000**, *21*, 832.
11. Sakurai, H.; Ritonga, M. T. S.; Shibatani, H.; Hirao, T. *J. Org. Chem.* **2005**, *70*, 2754.
12. (a) LeGourrierec, D.; Kharlanov, V. A.; Brown, R. G.; Rettig, W. *J. Photochem. Photobiol., A* **2000**, *130*, 101.
13. Rodembusch, F. S.; Leusin, F. P.; Campo, L. F.; Stefani, V. *J. Lumin.* **2007**, *126*, 728.
14. Balaji, G.; Shim, W. L.; Parameswaran, M.; Valiyaveettil, S. *Org. Lett.* **2009**, *11*, 4450.
15. (a) Akhtaruzzaman, M.; Tomura, M.; Nishida, J.; Yamashita, Y. *J. Org. Chem.* **2004**, *69*, 2953; (b) Akhtaruzzaman, M.; Tomura, M.; Zaman, M. B.; Nishida, J.; Yamashita, Y. *J. Org. Chem.* **2002**, *67*, 7813.
16. (a) Granzhan, A.; Ihmels, H. *Org. Lett.* **2005**, *7*, 5119; (b) Granzhan, A.; Ihmels, H.; Viola, G. *J. Am. Chem. Soc.* **2007**, *129*, 1254.
17. (a) Cardoso, M. B.; Samios, D.; da Silveira, N. P.; Rodembusch, F. S.; Stefani, V. *Photochem. Photobiol. Sci.* **2007**, *6*, 99; (b) Volkova, K. D.; Kovalska, V. B.; Losytskyy, M. Y.; Bento, A.; Reis, L. V.; Santos, P. F.; Almeida, P.; Yarmoluk, S. M. *J. Fluoresc.* **2008**, *18*, 877; (c) Shin, D. K.; Lee, S. C.; Heo, Y. S.; Lee, W. Y.; Cho, Y. S.; Kim, Y. E.; Hyun, Y. L.; Cho, J. M.; Lee, Y. S.; Ro, S. G. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5686.
18. Gauden, M.; Pezzella, A.; Panzella, L.; Neves-Petersen, M. T.; Skovsen, E.; Petersen, S. B.; Mullen, K. M.; Napolitano, A.; d'Ischia, M.; Sundstrom, V. *J. Am. Chem. Soc.* **2008**, *130*, 17038.
19. Czekster, C. M.; Neto, B. A. D.; Lapis, A. A. M.; Dupont, J.; Santos, D. S.; Basso, L. A. *Arch. Biochem. Biophys.* **2009**, *486*, 19.
20. LeGourrierec, D.; Kharlanov, V. A.; Brown, R. G.; Rettig, W. *J. Photochem. Photobiol., A* **2000**, *130*, 101.
21. (a) Corduneanu, O.; Chiorcea-Paquim, A. M.; Diculescu, V.; Fiuza, S. M.; Marques, M. P. M.; Oliveira-Brett, A. M. *Anal. Chem.* **2010**, *82*, 1245; (b) Lu, X. Q.; Zhu, K. M.; Zhang, M.; Liu, H. D.; Kang, J. W. *J. Biochem. Biophys. Methods* **2002**, *52*, 189.
22. (a) Shah, A.; Qureshi, R.; Khan, A. M.; Khera, R. A.; Ansari, F. L. *J. Braz. Chem. Soc.* **2010**, *21*, 447–451; (b) Aslanoglu, M. *Anal. Sci.* **2006**, *22*, 439.