PET modulated fluorescent sensing from the BF_2 chelated azadipyrromethene platform†

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Received 18th October 2005, Accepted 20th December 2005
First published as an Advance Article on the web 19th January 2006
DOI: 10.1039/b514788c

A convergent building block synthesis has been applied to new off/on photoinduced electron transfer (PET) modulated fluorescent sensors which are based on a BF_2 chelated tetraarylazadipyrromethene platform and operate in the biomedically important red region of the visible spectrum. Incorporation of diethylamine and morpholine receptors facilitates off/on microenvironment polarity and pH sensing. Aqueous formulation and *in vitro* cellular imaging demonstrates their potential for intracellular sensing.

Introduction

The development of molecular fluorescent sensors for biologically related applications is a very active research area.[1] The use of high sensitivity fluorescence detection has become a widely used tool for probing the molecular processes of biological systems *in vitro*,[2] and the application of fluorescence detection to non-invasive *in vivo* optical imaging is currently emerging.[3] The majority of fluorescent molecular sensors have operational light input/output wavelengths in the 300–550 nm wavelength range. In the case of *in vivo* imaging applications, this spectral region suffers strong interference from endogenous chromophores. In addition, fluorochrome excitation with blue and green light can be damaging to the cellular systems under observation. The most efficient light penetration of biological tissue occurs in the lower energy red and near-infrared (NIR) spectral regions.[4] As such, the development of new visible red and NIR off/on fluorescent sensors would be of benefit to future imaging applications. Several research groups have recently reported a number of visible red molecular sensors based on cyanines, squaraines and rhodamines, modified BODIPY dyes and *N*-phenyl-1,8-naphthalimides.[5]

Herein, we report a new class of fluorescent sensors based upon the BF_2 chelated tetraarylazadipyrromethene fluorophore **1** (Fig. 1) which employs a receptor–methylene spacer–fluorophore architecture. We have recently reported that this class of compound displays strong absorption and fluorescence in the 650–750 nm region of the spectrum.[6] For example, the parent tetraphenyl analogue of **1** (Ar^{1-4} = Ph) has an absorption λ_{max} at 650 nm (ε = 79 000 dm^{-3} mol^{-1} cm^{-1}) and emission at 672 nm (ϕ_F = 0.34) in chloroform. These promising photophysical characteristics suggest that these fluorophores would make an excellent platform from which visible red sensors could be constructed.

The design of fluorescent sensors most commonly adopts a receptor–spacer–fluorophore architecture with the fluorescence switching properties controlled by a photoinduced electron transfer (PET) mechanism.[7] The role of the receptor is to detect the targeted substrate, while the fluorophore quantifies and communicates this information to the observer. The spacer unit covalently links the fluorophore to the receptor whilst keeping the ground state electronic systems of the receptor and the fluorophore disconnected (Fig. 2). This sensing technology can be applied to a diverse set of substrates by the use of substrate specific receptors, allowing analytes such as protons, cations, anions, carbohydrates and peptides to be detected.[8]

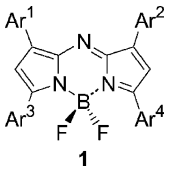

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Fig. 1 BF_2 chelated tetraarylazadipyrromethene fluorochromes.

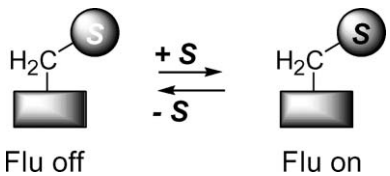

Fig. 2 Schematic of a PET fluorescent sensor design. Rectangle = fluorophore; CH_2 = methylene spacer; circle = receptor for substrate; **S** = substrate.

In order to examine the PET sensing properties of BF_2 chelated tetraarylazadipyrromethenes, we synthesised and examined the photophysical properties of the benzylamine substituted analogues **1a** and **1b**, which would have the potential to act as off/on switching receptors for pH[9] and microenvironment polarity (Fig. 3).[10]

The synthesis of our target compounds utilised our previously described modular approach, involving the synthesis and condensation of 2,4-diaryl-5-nitrosopyrroles with 2,4-diarylpyrroles.[11] Generation of **1a** and **1b** was accomplished through the condensation of 2-nitroso-3,5-diphenylpyrrole **2** with the receptor substituted pyrroles **3a,b** to yield the tetraarylazadipyrromethenes **4a,b**. BF_2 chelation of **4a,b** was achieved by reaction with boron-trifluoride diethyletherate with diisopropylethylamine as the base in dichloromethane at room temperature for 16 hours. Purification

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† Electronic supplementary information (ESI) available: ^1H and ^{13}C NMR spectra for **1a** and **1b**. UV–Visible and fluorescence solvent studies for **1a** and **1b**. See DOI: 10.1039/b514788c

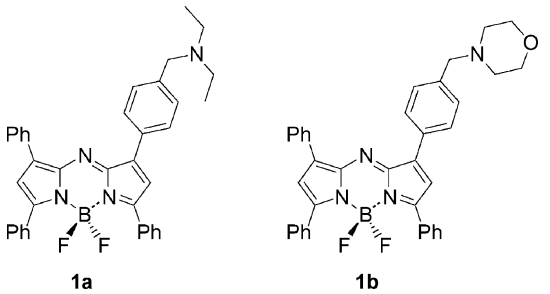


Fig. 3 PET modulated fluorescence molecular sensors.

of the final products was by chromatography on alumina, which provided **1a,b** as copper coloured solids in 88 and 91% yields respectively (Scheme 1).

Our expectations for the sensing properties of **1a,b** were that little change in the ground state UV–visible spectral properties would be observed on substrate recognition, however, large changes in the fluorescence intensity would act as the sole signalling event. The UV–visible spectra of **1a** in four solvents of decreasing dipolarity—DMF, THF, dioxane and cyclohexane—are shown in Fig. 4.[12] The wavelengths of the absorption maxima for **1a** show little solvent dependency with only a 10 nm hypsochromic shift observed for cyclohexane (647 nm) when compared to DMF (657 nm) (Table 1, Fig. 4). Identical behaviour was recorded for **1b** (Table 1, ESI†). Similarly, only a 12 nm difference between the wavelengths of the emission maxima in cyclohexane and DMF was observed for **1a** and **1b** which mirrored the minor hypsochromic shifts observed for non-polar solvents in the UV–visible spectra (Table 1).

Table 1 Spectral characteristics of **1a** and **1b** in nm

	DMF	THF	Dioxane	Cyclohexane
1a				
Abs[a]	657	653	652	647
Flu[b]	680	677	676	668
Abs/H^{+}[c]	657	655	653	650
Flu/H^{+}[c]	682	676	677	672
1b				
Abs[a]	656	654	652	647
Flu[b]	681	676	675	669
Abs/H^{+}[c]	657	656	654	652
Flu/H^{+}[c]	684	677	678	672

[a] Concentration of 1×10^{-5} M. [b] Concentration of 8×10^{-7} M, excitation 630 nm. [c] 5 μL of TFA added to a 3 mL sample.

In contrast to the invariance of the emission wavelength maxima in various solvents, the emission intensity showed a marked response to solvent polarity. The trend observed was that fluorescence intensity increased as a function of decreasing polarity along the series of DMF < THF < dioxane < cyclohexane (Fig. 5). A ninefold enhancement in fluorescence intensity was recorded when the extremes of DMF and cyclohexane were compared. It can be interpreted that in more polar solvents, little fluorescence is observed as the excited state quenching by the PET process is efficient (sensor is off). Whereas in non-polar solvents the PET process is an ineffective competing process for fluorescence emission and the sensor is switched on. The switching

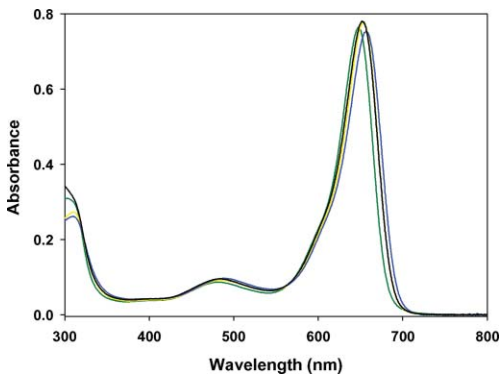


Fig. 4 UV–Visible spectra of **1a** in DMF (blue), THF (yellow), 1,4-dioxane (black), cyclohexane (green) at 1×10^{-5} M.

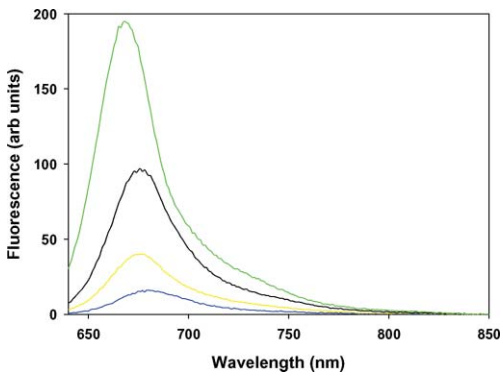
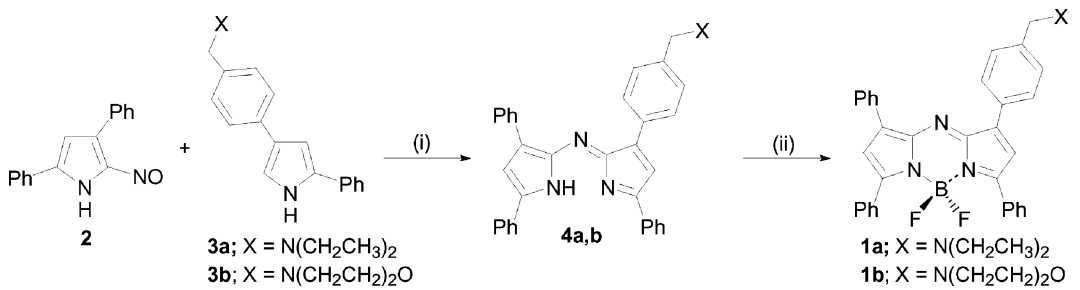


Fig. 5 Fluorescence spectra of **1a** in DMF (blue), THF (yellow), 1,4-dioxane (black) and cyclohexane (green). Concentration 8×10^{-7} M, excitation 630 nm, slit widths 5 nm.



Scheme 1 Synthesis of **1a** and **1b**. *Reagents and conditions*: (i) acetic anhydride, acetic acid, 100 °C, 1 h. (ii) $BF_3{\cdot}OEt_2$, DIEA, CH_2Cl_2, rt, 16 h.

effect of microenvironment polarity on the PET mechanism has been observed previously.[10b,13]

The one-dimensional switching of our sensors in response to an acid substrate (*i.e.* no significant ground state change in response to substrate recognition) is shown in Fig. 6. There is virtually no variance in the peak shape or the wavelength of maximum absorbance in the UV–visible spectra for protonated **1a** in any of the four solvents examined (Table 1, ESI). There is a marked response however to the acid analyte in the fluorescence spectra. For example the non-protonated **1a** is weakly emissive in DMF solutions but upon protonation with aqueous HCl a strong emission is observed at 682 nm. The increase in fluorescence intensity was greater than eightfold from the off to the on state (Fig. 6).

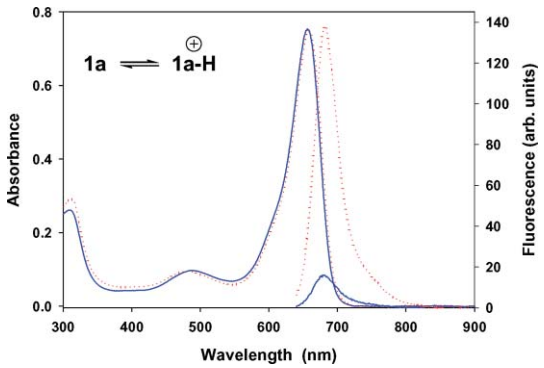


Fig. 6 Overlaid UV–visible (1×10^{-5} M) and fluorescence (8×10^{-7} M, excitation 630 nm, slit widths 5 nm) spectra of **1a** in DMF (blue) and in DMF–aq. HCl (red).

As would be expected, THF, dioxane and cyclohexane each gave smaller fluorescence intensity enhancements upon the addition of trifluoroacetic acid (ESI). A similar trend for off to on fluorescence enhancement (3-fold) in response to acidic conditions was observed for **1b** in DMF (ESI).

In order to obtain aqueous solutions suitable for *in vitro* imaging, the emulsifier Cremophor EL (CrEL) was used. CrEL is a non-ionic surfactant, frequently used *in vivo* as a delivery agent for poorly water-soluble drugs such as Taxol.[14] The aqueous formulated spectroscopic properties of **1a,b** were very similar to those obtained in organic solvents. The fluorescence titration of **1a** with aqueous HCl showed a significant increase in fluorescence intensity upon sequential addition of acid aliquots, whereas a more moderate increase was recorded for the morpholine receptor analogue **1b**. The titration data predicted apparent pK_a values of 6.9 and 4.8, for **1a** and **1b** respectively, providing a useful window of sensing under physiological conditions (Fig. 7, ESI). It should be noted that pK_a values of amines in a micellar microenvironment are often lower than would be anticipated.[15]

To examine the potential of our molecular sensor class for imaging *in vitro*, an aqueous formulated solution of **1a** was incubated with HeLa cells for 1 hour. The cells were washed of surface bound material, slide mounted and imaged using confocal laser scanning microscopy. Cellular localisation of emissive **1a** was examined through 16 focal plane sections of 0.48 μm apart through a single cell.[16] Analysis of the Z-stack plane views confirmed that emission from **1a** was exclusively in the cytoplasm (red) with no fluorescence observed in the nucleus (dark area) (Fig. 8).

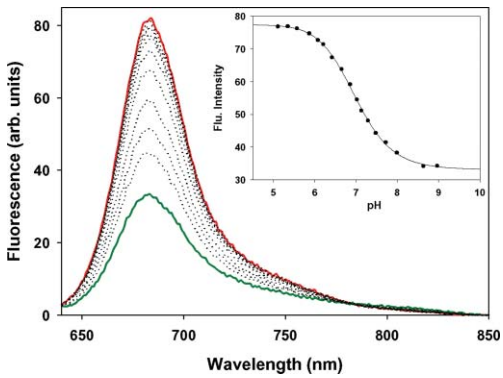


Fig. 7 Fluorescence titration of **1a** in H_2O/CrEL/phosphate buffered saline (I_{NaCl} = 150 mmol L^{-1}). Apparent pK_a determined to be 6.9 at 25 °C. Red trace pH = 4.9, dark green trace pH = 9. Concentration 8×10^{-7} M, excitation 630 nm, slit widths 5 nm.

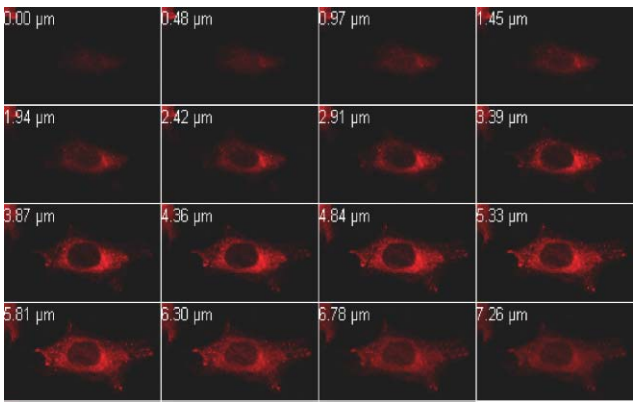


Fig. 8 Individual cellular focal plane sections of **1a** in a HeLa cell. Cytoplasm – red color; central dark area is the nucleus. Numbers in the left hand corner of each image indicate the cellular position at which the image was recorded.

Each image represents the fluorescence intensity at a specific focal plane of increasing depth through the cell. It can be seen that fluorescence intensity increases as you transect the cell (from 0 to 3 μM), reaches a maximum near the centre of the cell (~4 to 5 μM) and diminishes as the further edge of the cell is reached. In a cellular context **1a** has the potential to come into contact with a variety of different pH ranges and subcellular microenvironment polarities and as such the exact mode of intracellular switching is still under investigation.

Conclusions

We have developed a new fluorophore template for PET based sensing, using the receptor–methylene spacer–fluorophore approach from the boron-difluoro chelated azadipyrromethene platform, with an optical advantage of sensing in the red region of the spectrum. The flexible modular synthesis would readily allow for modified derivatives to be produced to include a range of receptor classes and to fine-tune any desired photophysical parameters. The ability of **1a** to localize and be readily detected *in vitro* gives an indication of the potential of this fluorochrome class as markers for specific cellular events in conjunction with other receptor units and fine-tuning of the switching responses. Future advances in the

field of fluorescent molecular sensing and imaging will provide new tools to assist in the understanding of biological processes at the molecular level.

Experimental

THF was distilled under N_2 over sodium wire and benzophenone. Cyclohexane, dioxane and chloroform were distilled over K_2CO_3. DMF was distilled under reduced pressure over K_2CO_3. Solutions for the solvent studies were prepared from a stock solution of **1** (0.005 mmol in 10 mL THF). 1 mL was diluted into 25 mL of either cyclohexane, DMF, dioxane or THF to provide a second stock solution. For UV–visible spectra, 5 mL of the second stock solution was diluted into 10 mL of the relevant solvent to give samples for UV–visible measurements. A 3 mL sample was removed and the UV–visible spectra recorded. 5 μL of trifluoroacetic acid (TFA) was added and the UV–visible spectra were recorded again. For fluorescence spectra 1 mL of the second stock solution was diluted into 25 mL of the relevant solvent to give samples for UV–visible measurements at 8×10^{-7} M. A 3 mL sample was removed and the fluorescence spectra were recorded. 5 μL of TFA was added and the fluorescence spectra were recorded again. Fluorescence measurements were recorded with the following setting; excitation and emission slit widths of 5 nm used, excitation wavelength 630 nm.

Synthesis of [3-(4-diethylamino-methylphenyl)-5-phenylpyrrol-2-ylidene]-(3,5-diphenyl-1*H*-pyrrol-2-yl)-amine BF_2 chelate 1a

A stirred solution of [3-(4-diethylamino-methylphenyl)-5-phenylpyrrol-2-ylidene]-(3,5-diphenyl-1*H*-pyrrol-2-yl)-amine[11] (107 mg, 0.2 mmol) in CH_2Cl_2 (50 mL) was treated with borontrifluoride diethyletherate (350 μL, 2.8 mmol) and diisopropylethylamine (350 μL, 2 mmol). The reaction was stirred at room temperature for 16 h, washed with water (2×50 mL), dried over Na_2SO_4, the solvent was removed under reduced pressure and the resulting solid was purified by chromatography on alumina (CH_2Cl_2–EtOAc, 4 : 1) to give the product **1a** as a copper colored solid (102 mg, 88%); mp 140–142 °C (cyclohexane); $\lambda_{max}(CHCl_3)$/nm 653 (ε/dm^{-3} mol^{-1} cm^{-1} 82 000); ν_{max}(KBr disk)/cm^{-1} 3446, 1513; δ_H(300 MHz; $CDCl_3$; Me_4Si) 1.08 (6H, t, *J* 7.1, $N(CH_2CH_3)_2$), 2.57 (4H, q, *J* 7.1, $N(CH_2CH_3)_2$), 3.63 (2H, s, CH_2N), 7.00 (2H, s), 7.43–7.47 (11H, m), 8.01–8.06 (8H, m); δ_C(75 MHz; $CDCl_3$; Me_4Si) 11.8, 46.9, 57.4, 118.8, 119.0, 128.6, 128.6, 129.1, 129.2, 129.4, 129.4, 129.6, 130.8, 130.9, 131.6, 131.7, 132.4, 141.8, 143.9, 144.3, 145.4, 145.8, 159.1, 159.8; *m/z* (ES) 583.2850 (M + H$^+$. $C_{37}H_{34}BF_2N_4$ requires 583.2845).

Synthesis of (3,5-diphenyl-1*H*-pyrrol-2-yl)-[3-(4-morpholin-4-ylmethylphenyl)-5-phenylpyrrol-2-ylidene]-amine BF_2 chelate 1b

A stirred solution of (3,5-diphenyl-1*H*-pyrrol-2-yl)-[3-(4-morpholin-4-ylmethylphenyl)-5-phenylpyrrol-2-ylidene]-amine[11] (107 mg, 0.2 mmol) in CH_2Cl_2 (50 mL) was treated with borontrifluoride diethyletherate (350 μL, 2.8 mmol) and diisopropylethylamine (350 μL, 2 mmol). The reaction was stirred at room temperature for 16 h, washed with water (2×50 mL), dried over Na_2SO_4, the solvent was removed under reduced pressure and the resulting solid was purified by chromatography on silica gel (EtOAc–cyclohexane, 2 : 1) to give the product as a copper colored solid (108 mg, 91%); mp 170–174 °C; $\lambda_{max}(CHCl_3)$/nm 652 (ε/dm^{-3} mol^{-1} cm^{-1} 82 000); ν_{max}(KBr disk)/cm^{-1} 3438, 1515; δ_H(300 MHz; $CDCl_3$; Me_4Si) 2.49 (4H, t, *J* 4.5, $N(CH_2CH_2)_2$), 3.55 (2H, s, CH_2N), 3.74 (4H, t, *J* 4.5, $(CH_2CH_2)_2O$), 7.00 (1H, s), 7.01 (1H, s), 7.40–7.48 (11H, m), 8.02–8.06 (8H, m); δ_C(75 MHz; $CDCl_3$; Me_4Si) 53.7, 63.2, 67.0, 118.9, 119.1, 128.6, 129.3, 129.4, 129.5, 129.5, 129.6, 129.6, 130.9, 131.2, 131.6, 132.3, 139.7, 144.0, 144.1, 145.5, 145.7, 159.4, 159.7; *m/z* (ES) 597.2634 (M + H$^+$, $C_{37}H_{32}BF_2N_4O$ requires 597.2637).

Formulation of 1a for *in vitro* cellular imaging

Compound **1a** (0.005 mmol) was dissolved THF (1 mL), Cremophor EL (0.07 mL) and 1,2-propanediol (0.03 mL) were added and the sample was placed in a sonic bath for 30 min. The THF was removed under reduced pressure and the oily mixture was dissolved in phosphate buffered saline (PBS) solution (10 mL) and filtered through an Acrodisc 25 mm syringe filter (with 0.2 μm HT Tuffryn membrane). The concentration was checked by diluting a portion of the sample (1 mL to 25 mL) with PBS and UV–visible spectral analysis.

Confocal laser scanning microscopy

Cells, grown on 8-well chamber slides (Nunc), were incubated in the dark at 37 °C with 1×10^{-5} M **1a** for 1 h. Prior to visualisation, excess probe was washed off by rinsing in PBS 4 times and cells were fixed in 3.7% formaldehyde–PBS. Cells were mounted as above and image analysis was performed using an LSM510 META confocal laser scanning microscope (Zeiss) equipped with a 40X numerical aperture 1.0 objective, with a pinhole of 100 μm in diameter being used to capture each image at a resolution of 512×512 pixels. **1a** was excited by a 543 nm helium neon laser.

Acknowledgements

This research was supported by the Program for Research in Third-Level Institutions administered by the HEA. Thanks to Dr D. Rai of the CSCB Mass Spectrometry Centre for analyses.

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