Water-solubilised BF_2-chelated tetraarylazadipyrromethenes†

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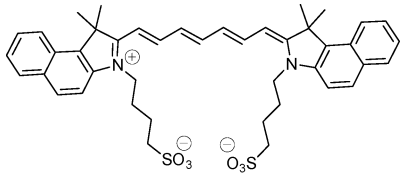
Received 18th September 2009, Accepted 31st October 2009
First published as an Advance Article on the web 16th November 2009
DOI: 10.1039/b919546g

Strategic incorporation of sulfonic acid, carboxylic acid or ammonium salt motifs generate water soluble BF_2-chelated tetraarylazadipyrromethenes which exhibit strong near infrared (NIR) emissions above 720 nm and can be readily imaged in both eukaryotic and prokaryotic cells.

High sensitivity NIR fluorescence imaging has become an indispensable tool for probing the molecular processes of biological systems in living cells. Its application to non-invasive *in vivo* animal and human imaging is currently a re-emerging field with applications varying from vascular mapping to tumour diagnosis.[1–3] NIR optical imaging of tissues is an inexpensive, real-time and non-invasive technique that does not require the use of radionuclides. Recently developed ultra sensitive low noise charge-coupled cameras, mathematical models of photon propagation in tissue, and more target-specific molecular probes have created exciting possibilities in this field.[4-6]

Several distinct advantages exist for fluorescence imaging in the NIR spectral region, such as increased light penetration of biological tissue, low auto-fluorescence of endogenous chromophores and minimal damaging of the cells/tissue under observation. Despite the optical benefits there is a surprising scarcity of NIR organic compounds which have the desired absorption and emission properties. To date, only indocyanine green (ICG) **1** has been approved for clinical use (Chart 1).[7]



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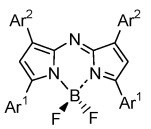

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Chart 1 Structure of indocyanine green **1** and BF_2-chelated tetraarylazadipyrromethene class **2**.

In spite of their poor photostability and lengthy synthetic routes, the cyanine dyes have been the most widely utilised class for applications in this spectral region.[8] Thus, an intensive effort has been put into synthesis of new NIR chromophores. Our recent efforts have focused on the boron chelated tetraarylazadipyrromethene class **2** as they are relatively easily synthesised, amenable to structural modification and exhibit excellent spectral properties.[9] Their strong absorption and emissions within the visible red/NIR spectral region, together with high photostability, make them promising candidates for biological imaging applications. We have previously shown that they are effectively imaged both *in vitro* and *in vivo* when delivered as formulated solutions.[9d,f] In addition, modulation of fluorescence intensity in response to specific stimuli such as pH, organic toxins and mercury ions has been achieved.[10]

In this report we present the synthesis of the first water solubilised BF_2-chelated tetraarylazadipyrromethenes, demonstrate their spectroscopic properties and *in vitro* delivery. We have previously reported that inclusion of an electron donating *para*-alkoxy group on the aryl rings α to the pyrrole nitrogen of **2** (Ar^1) results in significant emission bathochromic shift of ~40 nm when compared to the unsubstituted derivative.[9b] As such, this substitution pattern was included in the structural core of the fluorophore with additional carboxylic acid, sulfonic acid and ammonium salt functional groups introduced to provide aqueous solubility. Two strategies were adopted for the positioning of the solubilising groups onto **2**; the first included either sulfonic or carboxylic acid substituents as part of an alkoxy chain on Ar^1 and the second introduced ammonium salts into the *para* position of each of the β-aryl rings (Ar^2) with the Ar^1 rings having *para*-methoxy groups (Chart 1).

The synthesis of bis-carboxylic and bis-sulfonic acid functionalised derivatives had a common starting point of the bis-phenol substituted azadipyrromethene **3**, which is readily accessible from 1-(4-hydroxyphenyl)-3-phenylpropenone in two synthetic steps (Scheme 1).[11] Alkylation of both phenols of **3** with methyl bromoacetate gave the corresponding diester **4** in high yields, following purification by silica gel chromatography. Saponification of **5** with potassium trimethylsilanolate (TMSOK) in THF at room temperature afforded the bis-carboxylic acid derivative **6**. The optimised conditions to the bis-sulfonic acid analogue required BF_2 chelation of **3** to generate **7** and subsequent reaction with propane-1,3-sultone in presence of K_2CO_3 providing **8**, in moderate yield, as a dark green powder following chromatographic purification.[12]

The synthetic approach adopted for the bis-cationic derivative is outlined in Scheme 2. Heating of the 1,3-diaryl-4-nitrobutan-1-one **9**[9e] with ammonium acetate gave the azadipyrromethene **10** and subsequent BF_2 chelation under standard conditions generated **11** in a 72% yield. Reaction of **11** with methyl iodide at room temperature in dichloromethane proved an effective means to ensure complete alkylation with purified **12** obtained following recrystallisation from CH_2Cl_2–diethyl ether.[13]

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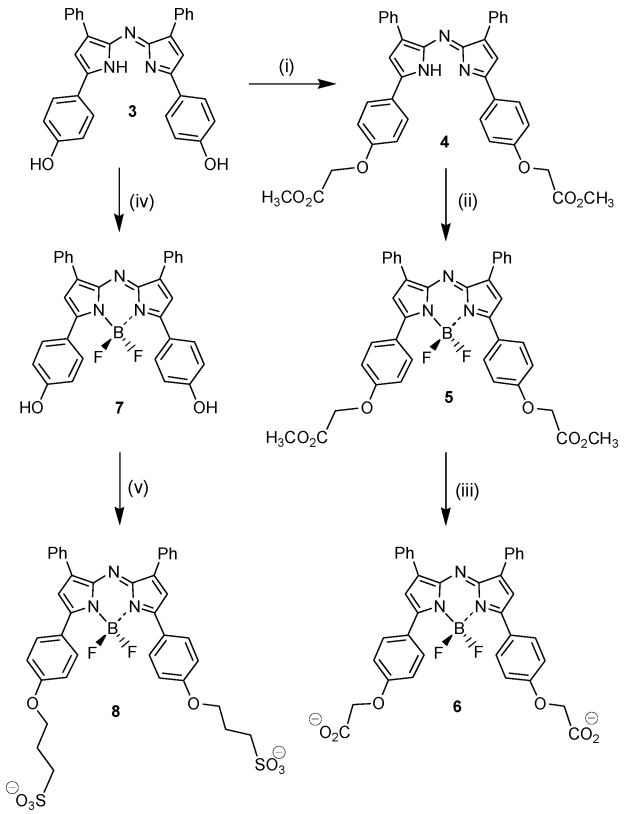
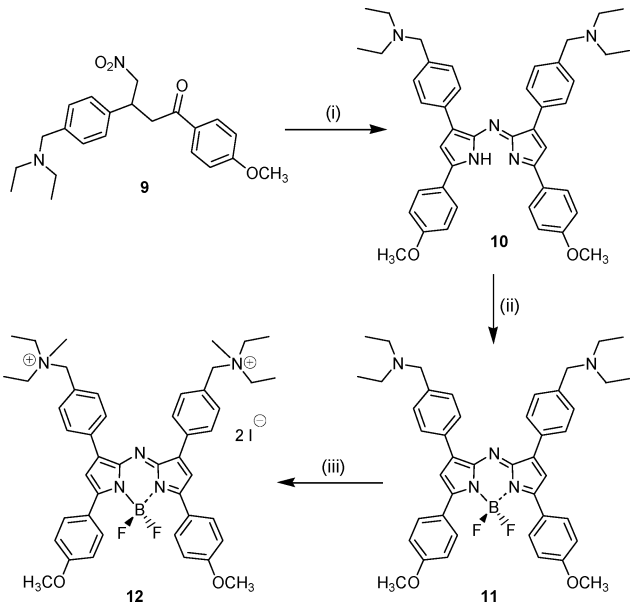
† Electronic supplementary information (ESI) available: Experimental procedures and characterisation data. See DOI: 10.1039/b919546g



Scheme 1 Synthesis of bis-anionic substituted derivatives. *Reagents and conditions*: (i) methyl bromoacetate, K_2CO_3, acetone, reflux, 16 h, 89%. (ii) $BF_3{\cdot}OEt_2$, DIEA, CH_2Cl_2, rt, 24 h, 73%. (iii) TMSOK, THF, rt, 3 h, 34%. (iv) $BF_3{\cdot}OEt_2$, DIEA, CH_2Cl_2, rt, 16 h, 78%. (v) propane-1,3-sultone, K_2CO_3, acetone, reflux, 6 h, 41%.



Scheme 2 Synthesis of bis-cationic substituted derivative. *Reagents and conditions*: (i) NH_4OAc, EtOH, reflux, 48 h, 36%. (ii) $BF_3.OEt_2$, DIEA, CH_2Cl_2, rt, 24 h, 72%. (iii) MeI, CH_2Cl_2, rt, 24 h, 80%.

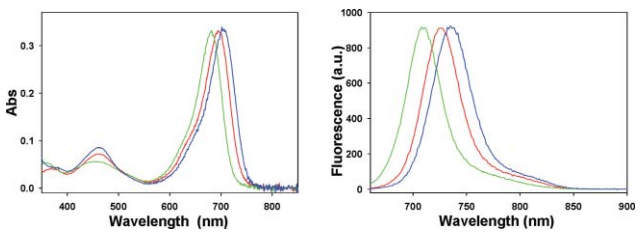


Fig. 1 Normalised absorption (left) and emission (right) spectra of **6** (green line), **8** (red line), and **12** (blue line) in $CHCl_3$.

Spectroscopic properties of **6**, **8** and **12** in organic solvents correspond very closely to those previously reported for this class of chromophore.[9b] For example in chloroform the absorption maxima range from 681 nm for **6**, 694 nm for **8**[14] to 702 nm for **12** with very minor shifts from these values recorded in methanol (Table 1, Fig. 1, ESI). Each fluorophore exhibited a strong fluorescence emission with quantum yields between Φ = 0.22–0.31 and maxima at 711, 726 and 735 nm for **6**, **8** and **12** respectively (Table 1, Fig. 1). Comparison of the three fluorophores showed only minor bathochromic shifts for the derivatives **6** and **8** when compared to **12** (Table 1).

As a representative biological aqueous solution, spectra of **6**, **8**, and **12** were taken in Dulbecco's modified Eagle's medium (DMEM) which is a commonly used medium for cellular manipulations.[15] Each fluorophore showed a small bathochromic shift in their λ_{max} of emission (5–12 nm) when compared to organic solvents, with emission bands extending from 700 to 800 nm (Fig. 2).

These spectroscopic properties correlate perfectly to the optical requirements of commercially available confocal laser scanning microscopy (CLSM) instruments and small animal optical imaging instruments.[16] Eukaryotic cellular uptake of DMEM solutions of **6** and **8** were utilised to illustrate their potential for *in vitro* imaging. Compounds **6** and **8** were incubated with MDA-MB-231 cells for 1 h at 5 μM concentration and the excess dye removed by washing with PBS before visualisation.

The obtained images showed that **6** and **8** were efficiently internalised by cells, after a relatively short incubation period, and were readily imaged by CSLM (Fig. 3a, 3b). 3-D Reconstruction

Table 1 Spectroscopic characteristics of **6**, **8** and **12**

		λ_{max}abs./nm[a]				λ_{max}emiss./nm[b,c,d]				
Entry	Comp.	$CHCl_3$ (ε)[e]	MeOH	DMEM	PBS–BSA	$CHCl_3$	MeOH	DMEM	PBS–BSA	Φ_f[f]
1	**6**	681 (89)	681	694	692	711	715	722	718	0.30
2	**8**	694 [g](51)	687	694	692	726	716	728	718	0.31
3	**12**	702 (69)	702	709	706	735	732	737	730	0.22

[a] Conc. 1×10^{-6} M. [b] Conc 5×10^{-7} M. [c] Excitation at 640 nm. [d] Slit widths 5 nm. [e] $\times 10^3$ M^{-1} cm^{-1}. [f] $CHCl_3$. [g] As tetrabutylammonium salt.

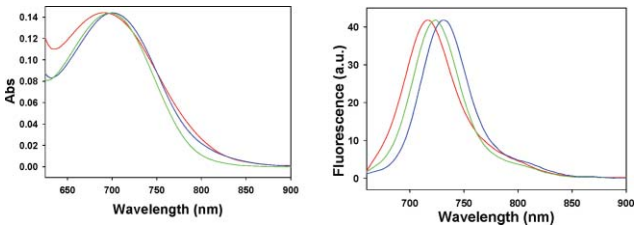


Fig. 2 Normalised absorption (left) and emission (right) spectra of **6** (green line), **8** (red line), and **12** (blue line) in DMEM. Absorption spectrum below 600 nm is not shown due to masking by phenol red dye contained in DMEM.

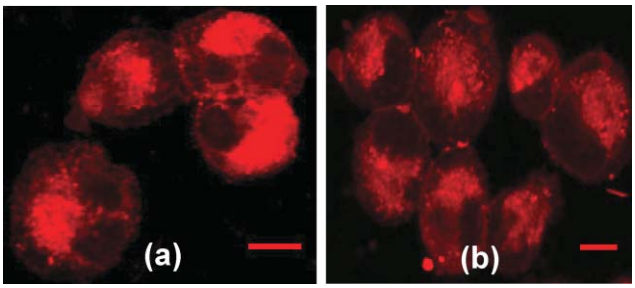


Fig. 3 CLSM images of fixed MDA-MB-231 cells after 1 h incubation with 5 μM solution of (a) **6** and (b) **8**. Scale bars, 10 μm.

of cellular distribution determined by the combination of 10 focal plane sections and nuclear co-staining with 4,6-diamidino-2-phenylindole (DAPI) showed that the subcellular localisation of **6** and **8** were primarily to the cytoplasm (ESI).

We envisaged that the bis-cationic nature of **12** would be optimal for uptake into prokaryotic cells thereby broadening the utility of this fluorophore class. Incubation of aqueous solutions of **12** with both gram-negative (*Escherichia coli*) or gram-positive (*Staphylococcus aureus*) bacterial cells for only 10 min was sufficient for efficient uptake (ESI). Confocal imaging confirmed the binding of **12** to both gram-positive and negative bacterial cells (Fig. 4a, 4b).

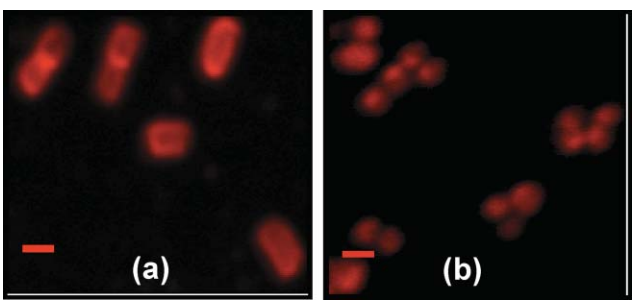


Fig. 4 CLSM images of (a) *E. coli* and (b) *S. aureus* cells after 10 min. incubation with 4 μM solution of **12**. Scale bars, 1 μm.

To establish the spectral characteristics of these fluorophores for potential *in vivo* imaging applications, we have examined their properties in the presence of serum proteins. Serum albumin is the most abundant protein in blood plasma at a typical concentration of ~50 g L^{-1}.[17] As one of its principal functions is to act as a binding and carrier protein within the vasculature it would be expected that strong interactions between it and our fluorophores could occur. This is known for ICG and the spectroscopic effects of plasma on ICG have been thoroughly documented.[18] As a preliminary examination to test if the spectral properties are adversely effected by such proteins we have recorded their spectra in aqueous BSA (bovine serum albumin) solutions. We observed sharp absorbance and emission bands in each case with emission maxima at 718 nm for **6** and **8** and 730 nm for **12** in a phosphate buffered saline (PBS) solution containing 4×10^{-4} M BSA (Fig. 5). These solutions remained stable for prolonged periods exposed to ambient light with less than 10% variance in absorbance and fluorescence intensity after 24 h (ESI). Collectively these properties are positive indicators for future use as *in vivo* NIR fluorophores.

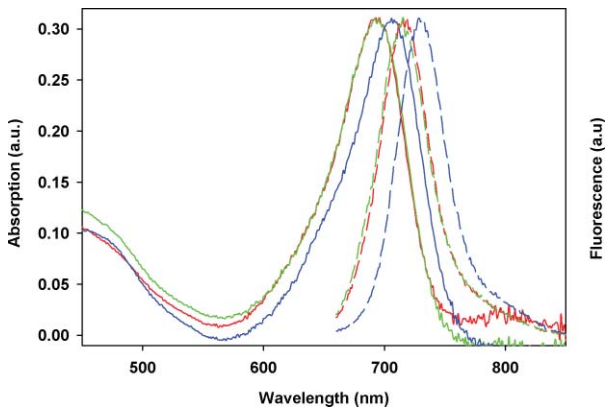


Fig. 5 Absorbance (solid line) and fluorescence (dashed line) spectra of **6** (green), **8** (red) and **12** (blue) in PBS–BSA solutions.

In summary, anionic and cationic substituted BF_2-chelated tetraarylazadipyrromethene derivatives, bearing sulfonic acid, carboxylic acid or quaternary amine moieties have been synthesised. These fluorophores show excellent photophysical characteristics in both organic and aqueous solutions. Delivery to and confocal imaging within eukaryotic and prokaryotic cells can be readily achieved. Their application to *in vivo* use is currently under investigation and will be reported upon in due course.

Acknowledgements

We would like to thank Science Foundation Ireland and The Irish Research Council for Science, Engineering and Technology for financial support.

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12 Synthesis of **8**. Compound **7** (168 mg, 320 mmol), propane-1,3-sultone (97 mg, 800 mmol) and K_2CO_3 (110 mg, 800 mmol) were heated under reflux in acetone (60 mL) for 6 h, under a N_2 atmosphere. The resulting precipitate was filtered, washed with acetone and cold methanol. Preparative RP-HPLC (C-18; acetonitrile–water, 60 : 40; retention time: 3 min) afforded **7** (102 mg, 41%) as a green solid m.p. > 300 °C. For, NMR analysis the compound was transformed into tetrabutylammonium salt by extraction of aqueous solution of **8** with $CHCl_3$ in presence of tetrabutylammonium chloride. The organic phase was washed with water twice, dried and evaporated. δ_H of **8**·$(NBu_4)_2$ (500 MHz, $CDCl_3$): 8.11–8.00 (m, 8H), 7.51–7.35 (m, 6H), 7.04 (s, 2H), 6.99 (d, J = 9.0, 4H), 4.25 (t, J = 6.4, 4H), 3.32–3.17 (m, 16H), 2.98 (t, J = 7.3, 4H), 2.42–2.28 (m, 4H), 1.61 (dt, J = 12.0, 7.8, 16H), 1.51–1.31 (m, 16H), 0.98 (t, J = 7.3, 24H). δ_C (100 MHz, $CDCl_3$): 161.7, 158.0, 145.2, 142.9, 132.5, 131.6, 129.21, 129.18, 128.5, 123.7, 118.6, 114.8, 67.4, 58.7, 48.2, 25.6, 23.9, 19.7, 13.7. HRMS (ESI) calcd for $C_{38}H_{33}BN_3O_8F_2S_2$ $[M - H^+]^-$ 772.1770, found 772.1757. IR (KBr disc) cm^{-1}: 1468, 1505, 1603.

13 Synthesis of **12**. Compound **11** (300 mg, 0.41 mmol) was dissolved in dry CH_2Cl_2 (60 mL), treated with methyl iodide (260 μL, 4.1 mmol) and stirred under N_2 for 24 h at rt. The solvent was removed under reduced pressure and recrystallisation from CH_2Cl_2–Et_2O (8 : 1) gave the product **12** (330 mg, 80%) as a dark green solid mp > 300 °C. δ_H (500 MHz, DMSO-d_6): 8.30 (d, J = 8.0 Hz, 4H), 8.20 (d, J = 9.0 Hz, 4H), 7.77 (d, J = 8.0 Hz, 4H), 7.74 (s, 2H), 7.17 (d, J = 9.0 Hz, 4H), 4.62 (s, 4H), 3.90 (s, 6H), 3.43–3.39 (m, 4H), 3.31–3.26 (m, 4H), 2.95 (s, 6H), 1.35 (t, J = 7.0 Hz, 12H). δ_C (125 MHz, DMSO-d_6): 162.7, 158.1, 145.1, 141.2, 133.8, 132.4, 129.7, 129.4, 123.4, 121.0, 115.0, 63.5, 56.1, 55.8, 46.6, 8.30. IR (KBr disc) cm^{-1}: 3434, 1603. ES-MS: m/z 884.7 $[M - I^-]^+$. HRMS (ESI) calcd for $C_{46}H_{54}BF_2IN_5O_2$ $[M - I^-]^+$ 884.3383, found 884.3381.

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