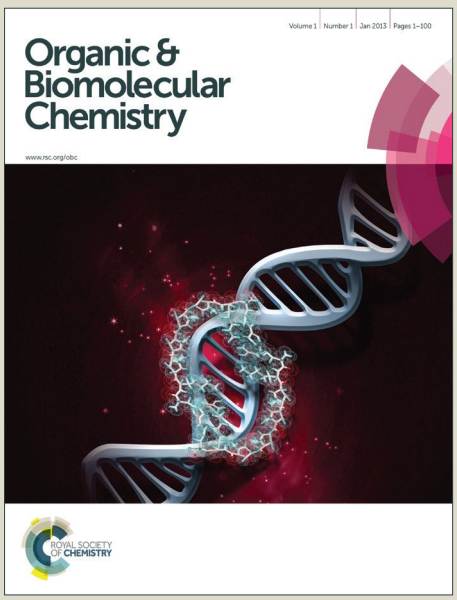
Organic & Biomolecular Chemistry

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Molecular Design and Synthesis of a pH Independent and Cell Permeant Fluorescent Dye and its Applications

Xiaojie Jiao,[a] Chang Liu,[a] Kun Huang,[a] Siwen Zhang,[a] Song He,[a] Liancheng Zhao[ab] and Xianshun Zeng*[a]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Fluorescent dyes have played crucial roles in the field of molecular imaging as fluorescent fluorophores. In this work, a novel water-soluble and pH-independent fluorescent xanthene dye, a hydroxyl regioisomeric 3',4'-benzorhodol has been designed and synthesized. Compared with those of rhodol dyes, the absorption (ca. 570 nm) and maximum emission (ca. 620 nm) of the dye are largely red-shifted. Due to its keeping a ring-opened zwitterion structure in water media, the dye showed good membrane permeability and distributed in whole cell cytoplasm upon incubation with live cells. Meanwhile, the dye could be easily modified to probes. The hydrazide derivative of the dye exhibited an excellent Hg^{2+} selectivity over other relevant metal ions and with a detection limit down to 3 nM. Thus, the excellent fluorescence properties and chemical properties of the dye allow it to be designed as fluorescent chemosensors and biomarkers for biologically related applications.

Introduction

The development of molecular fluorescent dyes is a continued attractive research topic in the fields of molecular sensing technology, molecular imaging technology and photonic and electronic devices.[1] For its high extinction coefficients, high quantum yields, and reasonable biocompatibilities, xanthene dyes, especially fluorescein and rhodamine derivatives, are the most popular fluorophores being widely employed as fluorescent probes in molecular biology, dye lasers and photosensitizers in dye-sensitized solar cells, etc.[1,2] However, fluorescein-based dyes have a few of deficiencies, such as irreversible photobleaching under the intense illumination,[3] pH-dependent fluorescence properties,[4] their protein conjugates exhibited quenched fluorescence relative to that of the free fluorophore in many cases,[5] etc. Rhodamine derivatives also exhibit strong tendency to form parallel π-π stacking dimers in concentrated solutions[6] or doped on solids,[7] resulted in emission quenching and low photosensitivity for their application.[8] The intrinsic deficiencies limited their applications as fluorescent labels and tracers. Thus, the design and synthesis of novel luminophores for biologically related applications is a continuously attractive research topic in recent years.

Since the sensitivity of fluorescent labels and tracers is proportional to the fluorophores' absorbance, and the quantum yield, the signaling fluorophores with high extinction coefficients, quantum yields, photostability, pH independent and solubility in a variety of solvents are usually required. With an unique combination structure of rhodamine and fluorescein, rhodol deravitives[9] (also called 'rhodafluor') are interesting candidates for fluorescent labels and tracers since they inherit all the excellent photophysical properties from fluorescein and rhodamine, such as high extinction coefficients, quantum yields, photostability, and solubility in a variety of solvents. Most of them showed stable fluorescent signals while the environmental pH is above 5.5. Recently, they have been successfully developed as a wide range of fluorescent tracers, including high reactive oxygen species, such as H_2O_2,[10] peroxynitrite,[11] and nitroxyl;[12] enzymes;[13] cysteine;[14] ions,[15,16] and other chemicals species.[17] However, to the best of our knowledge, the existing rhodol dyes are actually quite limited despite their excellent photophysical properties (Chart 1). Inspired by the relationships between the structures of rhodol derivatives and their excellent photophysical and photochemical properties, we assumed that the modification of the xanthene skeleton of rhodol will allow us to optimize the photophysical properties of rhodol dyes for the desired applications.

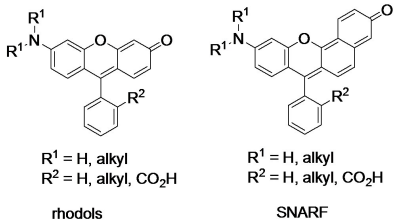
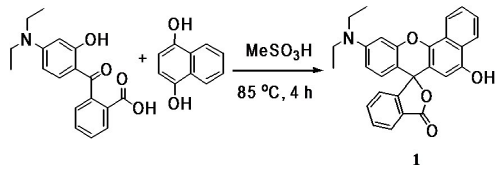


Chart 1. Structures of rhodols and SNARF.

We herein reported a 2'-hydroxyl-6'-(diethylamino)-3',4'-benzofluoran **1**, an unique xanthene based hydroxyl regio-isomeric 3',4'-benzorhodol dye, and also can be ascribed to a hydroxyl regioisomeric seminaphthorhodafluors (SNARF) (Chart 1).[18] The dye **1** revealed following advantages: 1) It is readily dissolved in water for it kept a ring-opened zwitterion structure in water, which is favorable for cell membrane permeability; 2) The maximum absorbance wavelength (ca. 570 nm) and emission

Organic & Biomolecular Chemistry Accepted Manuscript

maximum wavelength (ca. 620 nm) are largely red shifted, and with high extinction coefficients and moderate quantum yields (φ = 0.18); 3) The dye showed stronger tolerance to pH than rhodol (pKa ca. 5.5),[9] and it can generate stable optical signals in the pH range 4-9.5, such a pH tolerance meets the fluorescent labels or tracers for biologically related application, even in some special acidic organelles (such as lysosomes, endosomes, and phagosomes, where they had a pH of 5.0-6.5); 4) It can be easily chemical modified to yield all kinds of probes. Therefore, the intrinsic excellent fluorescence properties of the dye may allow it to be designed as fluorescent labels and tracers for biologically related applications.



1

Scheme 1. Structure and the Synthesis of the dye **1.**

Results and Discussion

Synthesis of the dye 1

The preparation of 2'-hydroxyl-6'-(diethylamino)-3',4'-benzofluoran **1** is illustrated in Scheme 1. Reaction of naphthalene-1,4-diol and commercially available 2-(4-diethyl-amino-2-hydroxybenzoyl)-benzoic acid in methanesulfonic acid afforded crude compound **1** as a red brown powder. The pure product was obtained as red powder in 87% yield by column chromatography. The structure of **1** was characterized by ESI-MS, ^{1}H- and ^{13}C-NMR, and elemental analysis.

Optical properties of the dye 1

Firstly, the photochemical properties of **1** in solvents with different polarities in the presence and absence of trifluoroacetic acid (TFA) were investigated by UV-Vis absorption and fluorescence measurements. The dye **1** in water solutions exhibited maxima absorption bands at 578 nm (ε = 3.81 × 10^4 M^{-1} cm^{-1}) that can be assigned to the π-π* transitions of the ring-opened 3,4-benzoxanthene conjugate. It also showed a moderate absorption at 568 nm (ε = 1.2 × 10^4 M^{-1} cm^{-1}) in the high polarity methanol solutions. No strong absorption was observed in other solvents (Fig. 1a). Upon addition of TFA (TFA/solvent, 1%, v/v), however, it immediately induced significant absorbance enhancement at ca. 580 nm with molar extinction coefficients between 3.0 × 10^4 M^{-1} cm^{-1} to 6.85 × 10^4 M^{-1} cm^{-1} in most of the solvents used (Table S1). Only a very weak increase of the absorbance at 578 nm (ε = 7.6 × 10^3 M^{-1} cm^{-1}) in dioxane was observed (Inset in Fig. 1a). At the same time, **1** showed a moderate to strong emission intensity (Φ1 = 0.18 in $CHCl_3$ in the presence of 1% TFA) positioned at ca. 620 nm in most of the solvents in the presence of TFA (Fig. 1b). However, the solvatochromism effects were observed both in absorption and fluorescence measurements with changing solvent polarity.[19] For instance, it showed a positive solvatochromism (red shift) in toluene, dichloromethane and chloroform and a negative solvatochromism (blue shift) in other solvents with high polarities. The results indicated that the spirolactone structure of **1** is stable in most organic solvents, but it is sensitive to acid. However, it is in a ring-opened π-conjugate form in both neutral and acidic water media. Then, a continuously pH titration of **1** in water revealed that it showed a strong absorption bands at 578 nm from pH 1.0 to 9.5 (Fig S1a). The absorbances at 578 nm remained stable from pH 4.0 to 9.5 (Fig S1b). It red shifted to 620 nm with the increase of the pH to 12. The pKa data of **1** derived from the titrations are 3.10 and 10.0 (Fig S1c and S1d), which correspond to the interconversions between cationic and neutral forms, and the interconversions between neutral and anionic forms, respectively (Scheme 2). A continuously fluorescence titrations also showed the same results (Fig S2a). The pKa derived from the fluorescence titrations are 3.4 and 10.1 (Fig S2c and S2d), which also correspond to the conversions between cationic and neutral forms, and the conversions between neutral and anionic forms, respectively (Scheme 2). Unlike rhodol derivatives[9,13a] showing a pKa ca. 5.5 and seminaphtho-fluoresceins (SNAFLs) and seminaphthorhodafluors(SNARFs) derivatives[18] exhibiting ratiometric fluorescence at two emission

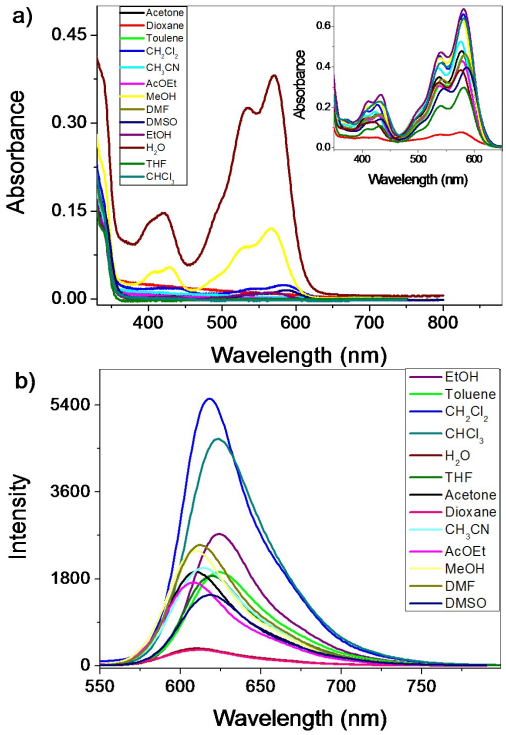
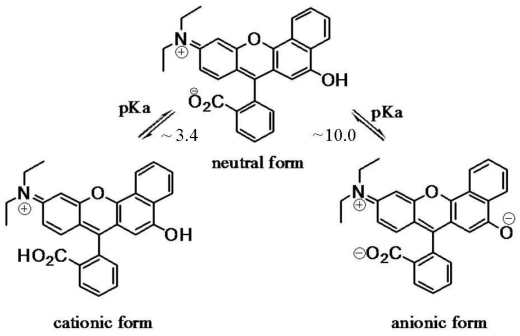


Figure 1. a) Absorption spectra of **1** (10 μM) in different solvents. Insets: **1** (10 μM) in different solvents in the presence of TFA (TFA/solvents, 1%, v/v). b) Emission spectra of **1** (10 μM) in different solvents in the presence of TFA (TFA/solvents, 1%, v/v). λ_{ex} = 540 nm. Slit: 5 nm; 5 nm.



Scheme 2. Representation of pH dependence equilibrium of **1**.

This journal is © The Royal Society of Chemistry [year]

Organic & Biomolecular Chemistry Accepted Manuscript

wavelengths (ca. 580 nm in acidic media and 630 nm in basic environments, respectively), **1** showed strong emission intensity positioned at ca. 620 nm from pH 1.0 to 9.5. Furthermore, the fluorescence signal is very stable from pH 4 to 9.5, implied that the pH independent properties meet the requirements of fluorescent labels or tracers for biologically related application, even in some special low pH organelles like lysosome, it can still produce stable signal.

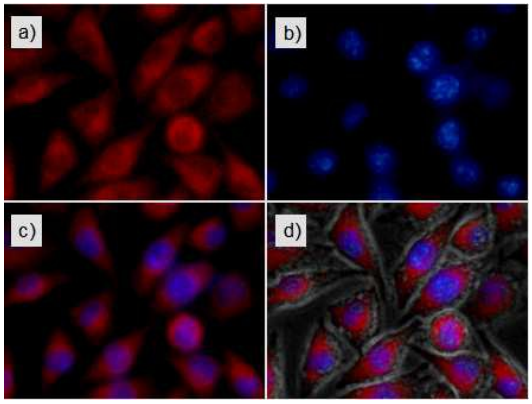


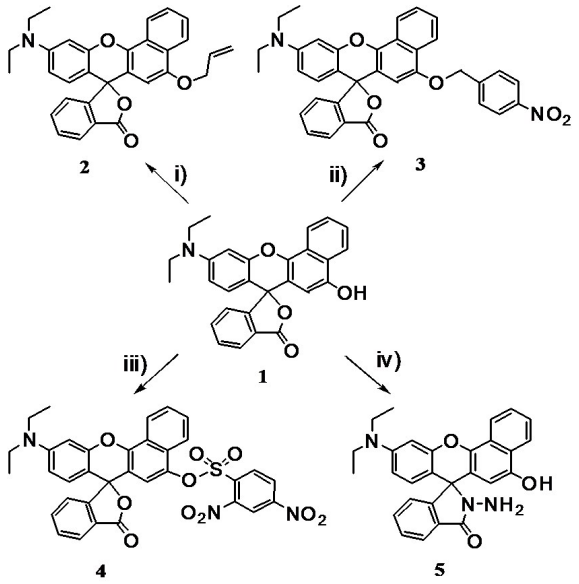
Figure 2. a) and b) fluorescence images of L929 cells incubated with the dye **1** (15 μM) and DAPI (0.1ug/ml), respectively. c) Overlay of images showing fluorescence from dye **1** and DAPI d) Overlay of bright-field, dye **1** and DAPI images.

Cell Permeability and fluorescence imaging of the dye 1

To elucidate the potential possibilities of **1** as signaling fluorophore in biologically related applications, the cell permeability of **1** was investigated by the incubation **1** with the L929 cell lines. After we incubated L929 cells with the dye **1** for 45 min in the growth medium, the fluorescence of the dye was present in the cells with an excitation of green light. By comparison with the bright-field, it is found that the fluorescence signals were localized in the perinuclear area of the cytosol, indicated the dyes were distributed in whole cell cytoplasm via multicolor fluorescent colocalization detection techniques (Fig. 2). It is interesting to note that the fluorescence of the dye was still present in the cells after 24 h (Fig. S3), indicated that the dye was kept in the cell without escaping from cells or decomposed by the cells. Bright-field measurements after 24 h confirmed that the cells were viable throughout the imaging experiments (Fig. S3c). The results indicate that the dye has good membrane permeability, low toxicity, and good biocompability and can be used as potential signaling fluorophore for the molecular design of biolabels for live cell imaging.

Chemical modifications of the dye 1

Then, we investigated the possibilities of the chemical modification of **1** to get a general knowledge of its chemical properties (Scheme 3). We have tried two approaches to modify the dye **1**. One is the direct modification of hydroxyl group. Compounds **2** and **3** were obtained in reasonable yields via alkylation of the hydroxyl group with corresponding bromides in acetonitrile under refluxed temperature in the presence of K_2CO_3 as acid-bonding reagent. Compound **4** was obtained by the reaction of **1** with 2,4-dinitro-benzenesulphonyl chloride. Another approach is modification of the carboxyl group by the reaction of **1** with hydrazine monohydrate, a general approach has been widely used for the preparation of rhodamine derivatives.[1] Thus, hydrazide **5** is facilely obtained in 64% yield by refluxing a mixture of **1** and hydrazine monohydrate in ethanol. The structures of **2-5** were confirmed by their ^1H, ^{13}C-NMR and ESI-MS spectra and elemental analysis. The results indicated that the dye can be easily modified by chemical approaches, which are of paramount importance properties for the molecular design of the dye as signaling fluorophore for chemosensors and biologically related applications.



Scheme 3. Chemical modifications of **1**. Reagents and conditions: i) 3-bromopropene/K_2CO_3, CH_3CN, refluxed for 32 h; ii) 4-chloromethylnitro-benzene/K_2CO_3, CH_3CN, refluxed for 28 h; iii) 2,4-dinitrobenzene-sulfonyl chloride/NEt_3, CH_2Cl_2, 48 h at rt; iv) $H_2NNH_2{\cdot}H_2O$, EtOH, refluxed for 48 h.

The molecular recognition properties of the hydrazide 5 toward Hg^{2+} ions

Finally, the molecular recognition properties of the hydrazide **5** toward Hg^{2+} ions were investigated in details. Fig. 3a shows the spectroscopic properties of **5** at varied concentrations of Hg^{2+} ions. As expected, the hydrazide **5** displays a colorless solution and emits no fluorescence. Addition of Hg^{2+}, however, it immediately produced a light purple color with an absorption maximum at 576 nm ($\varepsilon = 3.16 \times 10^4$ M^{-1} cm^{-1}) (Fig. S4) and emission maximum at ca. 621 nm (Fig. 3a), which closely resembles the spectroscopic features of **1**. This dramatic change of color in the presence of Hg^{2+} suggests that **5** would be a practical 'naked-eye' chemosensor of Hg^{2+} in aqueous solutions (Fig. S5). The resulting absorbance and fluorescence signal remain constant for at least 24 h. The mass spectra of the mixture **5** and Hg^{2+} showed clear peaks (m/z) of **5** and its hydrolyzed product of **1** at 452.1981 $[\mathbf{5} + H]^+$ and 438.1716 $[\mathbf{1} + H]^+$, respectively, which suggested that the selective optical responsive mechanism was the Hg^{2+}-promoted hydrolysis (Fig. S6). The increase of the fluorescence intensity at 621 nm followed the sigmoidal curves and the fluorescence turn-on constant ($K_{turn\text{-}on}$) was calculated as 77.24 ± 0.93 μM (with correlation coefficient R

Organic & Biomolecular Chemistry Accepted Manuscript

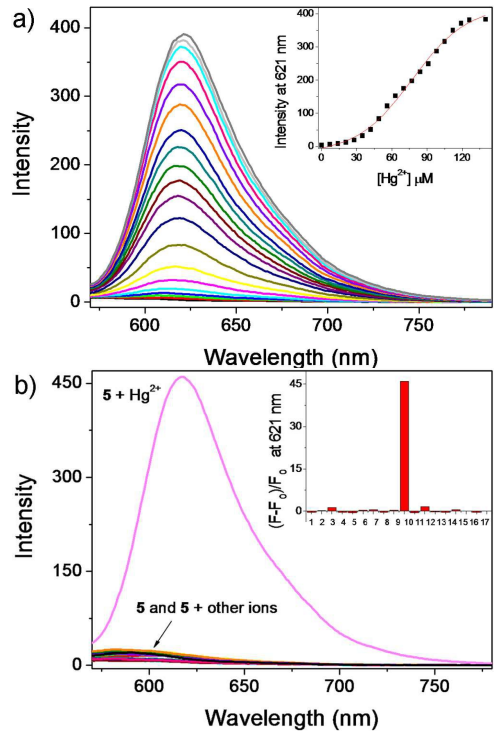


Figure 3. a) Fluorescent spectra of **5** (10 μM) in the presence of different concentrations of $Hg(NO_3)_2$ in ethanol/water (1:3, v/v). Inset: the fluorescence changes as a function of the Hg^{2+} concentration. b) Fluorescence spectra of **5** (10 μM) upon the addition of the nitrate salts (5 equiv.) of cations (1: Ag^+, 2: Al^{3+}; 3: Ca^{2+}, 4: Cd^{2+}, 5: Co^{2+}, 6: Cr^{3+}, 7: Cu^{2+}, 8: Fe^{2+}, 9: Fe^{3+}, 10: Hg^{2+}, 11: K^+, 12: Mg^{2+}, 13: Na^+,14: NH_4^+, 15: Ni^{2+},16: Pb^{2+}, and 17: Zn^{2+}) in ethanol/H_2O (1:3, v/v). Inset: histogram representing the fluorescence enhancement of 5 in the presence of metal ions. λ_{ex} = 560 nm. Slit: 5 nm; 5 nm.

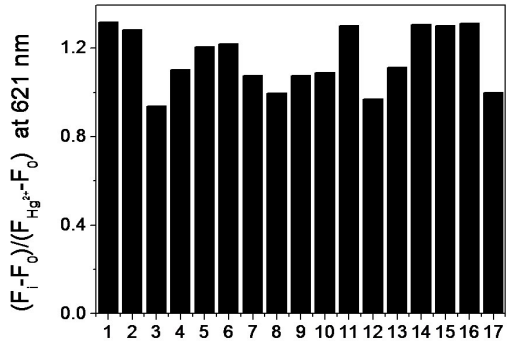


Fig. 4 Change ratio $((F_i-F_0)/(F_{Hg^{2+}}-F_0)$ of fluorescence intensity of **5** (10 μM) at 621 nm in various mixtures of metal ions ($Hg(NO_3)_2$ (50 μM) and one other metal ions (50 μM) in ethanol / water (v / v; 1:3)). 1: Hg^{2+} + Ag^+; 2: Hg^{2+} + Al^{3+}; 3: Hg^{2+} + Ca^{2+}; 4: Hg^{2+} + Cd^{2+}; 5: Hg^{2+} + Co^{2+}; 6: Hg^{2+} + Cr^{3+}; 7: Hg^{2+} + Cu^{2+}; 8: Hg^{2+} + Fe^{2+}; 9: Hg^{2+} + Fe^{3+}; 10: Hg^{2+} + K^+; 11: Hg^{2+} + Mg^{2+}; 12: Hg^{2+} + Na^+; 13: Hg^{2+} + NH_4^+; 14: Hg^{2+} + Ni^{2+}; 15: Hg^{2+} + Pb^{2+}; 16: Hg^{2+} + Zn^{2+}; 17: Hg^{2+}.

= 0.996).[20] From the Hg^{2+}-dependent fluorescence changes (Fig. S7), the detection limit was estimated to be 2.89 nM, indicating that the limit of detection of **5** for Hg^{2+} met the US EPA specified threshold limit for mercury content in drinking water (2 ppb, 10 nM).[21] Based on the Job's plot analysis, the spirolactam **5** reacted with Hg^{2+} in a 1 : 1 ratio as expected (Fig. S8). Subsequently, we investigated the response of **5** to other cations. The addition of 5.0 equivalents of a series of cations has no obvious effect on the UV-Vis absorption (Fig. S9) and fluorescence emission properties (Fig. 3b). In contrast, the addition of Hg^{2+} resulted in a prominent absorption enhancement at 576 nm and a significant enhancement of the emission intensity at 621 nm; thus, **5** can function as a highly selective optical probe for the Hg^{2+} cations. At the same time, the competition experiments showed no strong interference on the detection of Hg^{2+} ions in the presence of 5.0 equivalents of a series of cations (Fig. 4). The results demonstrated that hydrazide **5** is able to discriminate between Hg^{2+} and chemically close ions, especially Ag^+ ions which are common interfering ions or co-response ions in many cases are eliminated.[22]

Conclusion

In conclusion, we have developed a novel xanthene dye from commercially available naphthalene-1,4-diol and 2-(4-diethylamino-2-hydroxybenzoyl)benzoic acid. It exhibits a combination of desirable characteristics for chemsensors and biomarkers including (i) low molecular weight, (ii) water solubility, (iii) cell permeability, (iv) good biocompability, and (v) strong tolerance to pH. Meanwhile, the dye can be easily modified to chemosensors and biomarkers. We are now exploring the use of the dye as fluorophore in applications requiring pH tolerance bioprobes.

Experimental

General methods

Starting materials and reagents were purchased from Tokyo Kasei Kogyo (TCI: Tokyo, Japan), AR grade or dry grade solvents were purchased from Alfa-Aesar, and used without further purification. The reactions were carried out in oven-dried glasswares with a magnetic stirring. NMR spectra were recorded on a Bruker spectrometer at 400 (^{1}H NMR) MHz and 100 (^{13}C NMR) MHz. Chemical shifts were reported in ppm down field from internal Me_4Si (^{1}H and ^{13}C NMR). High resolution mass spectra (HRMS) were acquired on an Agilent 6510 Q-TOF LC/MS instrument (Agilent Technologies, Palo Alto, CA) equipped with an electro-spray ionization (ESI) source. Elemental analyses were performed on a Vanio-EL elemental analyzer (Analyze-system GmbH, Germany). Melting points were recorded on a Boethius Block apparatus and uncorrected. All absorption spectra were recorded using a Shimadzu UV-2550 UV/Vis spectrophotometer with 1cm quartz cell. In a similar manner, fluorescence spectra were recorded on a Hitachi F-4600 spectrofluorophotometer with a 1 cm quartz cell. Cells imaging was performed with a Nikon Eclipse TE2000U inverted fluorescence microscopy.

The preparation of 2'-hydroxyl-6'-(diethylamino)-3',4'-benzofluoran 1.

2-Hydroxy-4-diethylamino-2'-hydroxycarbonylbenzophenone (3.133 g, 10.0 mmol), naphthalene-1,4-diol (1.756 g, 11.0 mmol), methanesulfonic acid (10 mL) were added to a 100 mL flask. The reaction mixture was stirred for 4 h at 85 °C. Then 70 mL water was added into the reaction mixture, neutralized with $NaHCO_3$, and then extracted with ethyl acetate. The combined organic

Organic & Biomolecular Chemistry Accepted Manuscript

This journal is © The Royal Society of Chemistry [year]

phase was dried over Na_2SO_4. After filtration, the filtrate was condensed to dryness. The residue was recrystallized from methanol. The product was obtained as a red powder (3.820 g) in 87% yield; m.p. 256-258 °C; HRMS (LC/MS) m/z: $[M + H]^+$ = 438.1705, cacld: 438.1705; ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 9.93 (s, 1H), 8.54 (d, 1H, *J* = 8.0 Hz), 8.14 (d, 1H, *J* = 7.6 Hz), 8.07 (td, 1H, *J* = 7.6 Hz, 1.2 Hz), 7.77 (dd, 1H, *J* = 8.0 Hz, 1.2 Hz), 7.71 (dd, 1H, *J* = 8.0 Hz, 1.2 Hz), 7.65 (td, 1H, *J* = 8.0 Hz, 1.2 Hz), 7.30 (d, 1H, *J* = 7.6 Hz), 6.72 (d, 1H, *J* = 2.4 Hz), 6.56 (d, 1H, *J* = 8.8 Hz), 6.49 (dd, 1H, *J* = 8.8 Hz, 2.4 Hz), 6.06 (s, 1H), 3.38 (m, 4H, *J* = 6.8 Hz), 1.12 (t, 6H, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 169.89, 153.95, 153.05, 150.16, 149.73, 140.68, 136.57, 131.04, 129.59, 128.09, 127.90, 127.15, 126.75, 125.52, 125.18, 125.13, 123.19, 122.70, 113.47, 109.76, 104.93, 104.43, 98.06, 85.20, 44.72, 13.31; Anal. calcd for $C_{28}H_{23}NO_4 \cdot H_2O \cdot$MeOH; C, 73.83; H, 5.53; N, 3.08; Found: C, 73.65; H, 5.57; N, 2.91%.

The preparation of 2'-(dibenzylamino)-6'-allyloxyl-7',8'-benzofluoran 2.

To a 50 mL flask, was added **1** (436 mg, 1.0 mmol), K_2CO_3 (276 mg, 2.0 mmol), CH_3CN (20 mL) and 3-bromopropene (484 mg, 4.0 mmol). The reaction mixture was refluxed for 32 h under nitrogen atmosphere. The solvent was removed under reduced pressure. The residue was dissolved in water (20 mL) and dichloromethane (20 mL). The organic layer was separated, and the water phase was extracted with dichloromethane (10 mL × 2). The combined organic phase was dried with anhydrous Na_2SO_4 and then condensed to dryness. The residue was purified by column chromatography (SiO_2, AcOEt/CH_2Cl_2, v/v, 1:5). Compound **2** was obtained as purple powder in 56 % yield; m.p. 176-178 °C; HRMS (LC/MS) m/z: $[M + H]^+$ = 478.2012, calcd: 478.2018; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 8.58 (d, 1H, *J* = 7.6 Hz), 8.26 (d, 1H, *J* = 8.0 Hz), 8.07 (dd, 1 H, *J* = 8.0 Hz, 1.6 Hz), 7.63-7.70 (m, 4H), 7.16 (dd, 1H, *J* = 6.4 Hz, 1.6 Hz), 6.65 (d, 1H, *J* = 8.8 Hz), 6.63 (d, 1H, *J* = 2.4 Hz), 6.42 (dd, 1H, *J* = 8.8 Hz, 2.4 Hz), 6.00 (s, 1H), 5.97 (dddd, 1H, *J* = 17.3 Hz, 5.6 Hz, 3.8 Hz, 1.0 Hz), 5.99 (dddd, 1H, *J* = 17.3 Hz, 10.5 Hz, 1.4 Hz, 1.2 Hz), 5.19 (dddd, 1H, *J* = 10.5 Hz, 1.4 Hz, 1.2 Hz, 1.0 Hz), 4.45 (dddd, 1H, *J* = 12.6 Hz, 3.8 Hz, 1.4 Hz, 1.2 Hz), 4.34 (dddd, 1H, *J* = 12.6 Hz, 5.6 Hz, 1.4 Hz, 1.2 Hz), 3.40 (m, 4H, *J* = 6.8 Hz), 1.21 (t, 6H, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 170.12, 154.10, 152.92, 150.24, 149.81, 142.25, 135.11, 133.31, 129.76, 129.13, 127.39, 127.33, 127.28, 125.30, 125.12, 124.43, 122.45, 117.99, 112.23, 109.08, 105.10, 102.46, 98.03, 85.45, 69.56, 44.80, 12.88; Anal. calcd for $C_{31}H_{27}NO_4 \cdot 5/2H_2O$; C, 71.25; H, 6.17; N, 2.68; Found: C, 71.55; H, 6.07; N, 2.91%.

The preparation of 2'-(dibenzylamino)-6'-(4''-nitrobenzyloxyl)-7',8'-benzofluoran 3.

To a 50 mL flask, was added **1** (436 mg, 1.0 mmol), K_2CO_3 (276 mg, 2.0 mmol), CH_3CN (15 mL) and 4-chloromethylnitrobenzene (275 mg, 1.5 mmol). The reaction mixture was stirred for 28 h at 85 °C under nitrogen atmosphere. The solvent was evaporated to dryness. The solid residue was dissolved in water (15 mL) and dichloromethane (30 mL). The organic layer was separated, and the water phase was extracted with dichloromethane (20 mL × 2). The combined organic phase was dried with anhydrous Na_2SO_4 and then condensed to dryness. The residue was purified by column chromatography (SiO_2, AcOEt/CH_2Cl_2, v/v, 1:5). The product was obtained as a faint yellow powder (490 mg) in 86% yield; m.p. 268-272 °C; HRMS (LC/MS) m/z: $[M + H]^+$ = 573.2024, cacld: 573.2026; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 8.58 (d, 1H, *J* = 8.0 Hz), 8.27 (d, 1H, *J* = 8.0 Hz), 8.10 (d, 2H, *J* = 8.4 Hz), 8.02 (d, 1H, *J* = 7.4 Hz), 7.69 (td, 1H, *J* = 8.0 Hz, 1.2 Hz), 7.64 (td, 1H, *J* = 7.4 Hz, 1.2 Hz), 7.57 (td, 1H, *J* = 7.4 Hz, 1.2 Hz), 7.52 (td, 1H, *J* = 8.0 Hz, 1.2 Hz), 7.43 (d, 2H, *J* = 8.4 Hz), 7.01 (d, 1H, *J* = 7.4 Hz), 6.61 (d, 1H, *J* = 8.8 Hz), 6.60 (d, 1H, *J* = 2.0 Hz), 6.40 (dd, 1H, *J* = 8.8 Hz, 2.0 Hz), 5.88 (s, 1H), 5.13 (d, 1H, *J* = 13.2 Hz), 4.93 (d, 1H, *J* = 13.2 Hz), 3.39 (m, 4H, *J* = 6.8 Hz), 1.20 (t, 6H, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 170.13, 154.02, 152.72, 149.80, 149.41, 147.63, 144.56, 142.52, 135.13, 129.77, 129.13, 127.73, 127.57, 127.01, 126.93, 125.36, 125.13, 124.26, 124.06, 122.63, 122.23, 111.93, 109.12, 104.67, 102.98, 97.86, 85.27, 69.27, 44.82, 12.86; Anal. calcd for $C_{35}H_{28}N_2O_6$; C, 73.41; H, 4.93; N, 4.89; Found: C, 73.52; H, 4.72; N, 4.87%.

The preparation of 2'-(dibenzylamino)-6'-(2'',4''-dinitrobenzenesulfonyloxyl)-7',8'-benzofluoran 4.

To a 50 mL flask, was added **1** (436 mg, 1.0 mmol), anhydrous CH_2Cl_2 (15 mL), triethylamine (300 uL). A solution of 2, 4-dinitrobenzenesulfonyl chloride (0.329 g, 1.2 mmol) in anhydrous CH_2Cl_2 (5 mL) was added dropwise to the solution. The resulted reaction mixture was stirred for 48 h at room temperature. Then, the mixture liquid was washed with water (20 mL × 2). The combined organic phase was dried with anhydrous Na_2SO_4 and then condensed to dryness. The residue was purified by column chromatography (SiO_2, AcOEt/CH_2Cl_2, v/v, 1:10). Compound **4** (570 mg) was obtained as a brick-red solid in 85 % yield, m.p. 198-202 °C; HRMS (LC/MS) m/z: $[M + H]^+$ = 668.1336, calcd: 668.1339; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ major component: 8.66-8.62 (m, 1H), 8.54 (d, 1H, *J* = 2.16 Hz), 8.39 (dd, 1H, *J* = 8.6 Hz, 2.0 Hz), 8.18-8.15 (m, 1H), 7.90 (d, 1H, *J* = 8.28 Hz), 7.88 (dd, 1H, *J* = 8.28 Hz, *J* = 2.16 Hz), 7.74 (d, 1H, *J* = 7.6 Hz), 7.73 (t, 1H, *J* = 7.6 Hz,), 7.63 (dd, 1H, *J* = 7.6 Hz, 1.2 Hz), 7.58 (td, 1H, *J* = 7.6 Hz, 1.2 Hz), 7.10 (d, H, *J* = 7.6 Hz), 6.63 (d, H, *J* = 2.4 Hz), 6.56 (d, H, *J* = 8.8 Hz), 6.43 (dd, H, *J* = 8.8 Hz, 2.4 Hz), 6.10 (s, 1H), 3.40 (m, 4H, *J* = 7.2 Hz), 1.21 (t, 6H, *J* = 7.2 Hz); Minor component: 8.83 (d, 1H, *J* = 2.72 Hz), 8.72 (d, 1H, *J* = 8.4 Hz), 8.19-8.17 (m, 1H), 8.01 (d, 1H, *J* = 7.44 Hz), 7.81 (d, 1H, *J* = 8.32 Hz), 7.76 (d, 1H, *J* = 7.6 Hz,), 7.71 (dd, H, *J* = 7.6 Hz, 2.0 Hz), 7.68 (td, H, *J* = 7.6 Hz, 1.2 Hz), 7.65 (dd, H, *J* = 7.6 Hz, 1.2 Hz), 7.19 (d, H, *J* = 7.6 Hz), 6.77 (d, H, *J* = 9.2 Hz), 6.68 (d, H, *J* = 2.4 Hz), 6.65 (d, H, *J* = 8.8 Hz), 6.59 (s, 1H), 6.47 (dd, H, *J* = 8.8 Hz, 2.4 Hz), 3.43 (m, 2H, *J* = 7.2 Hz), 3.15 (m, 2H, *J* = 7.2 Hz), 1.36 (t, 3H, *J* = 7.2 Hz), 1.23 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 169.23, 165.26, 152.87, 152.46, 151.52, 150.01, 148.85, 147.12, 140.83, 135.57, 134.45, 132.84, 130.35, 129.43, 129.22, 128.35, 128.30, 128.25, 126.91, 126.71, 125.71, 125.09, 124.41, 123.61, 123.03, 122.39, 120.44, 117.55, 116.34, 115.79, 112.24, 109.82, 998.11, 77.56, 46.63, 45.01, 12.81; Anal. calcd for $C_{34}H_{25}N_3O_{10}S \cdot H_2O$; C, 59.56; H, 3.97; N, 6.13; Found: C, 59.56; H, 4.15; N, 5.86%.

Organic & Biomolecular Chemistry Accepted Manuscript

Preparation of the hydrazide 5.

To a 100 mL flask, was added **1** (2.99 g, 6.86 mmol), anhydrous ethanol (20 mL) and hydrazine monohydrate (5 mL). The reaction mixture was refluxed for 48 h under nitrogen atmosphere. After cooling to room temperature, the compound **5** was precipitated as a light yellow powder from the solution. The product was collected by filtration and washed with anhydrous ethanol (3 mL × 2). The product was obtained as a light yellow powder (1.9 g) in 64% yield; m.p. 264-268 °C; HRMS (LC/MS) m/z: $[M + H]^+$ = 452.1974; cacld：452.1974; ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 9.77 (s, 1H), 8.49 (d, 1H, *J* = 8.0 Hz), 8.11 (d, 1H, *J* = 8.0 Hz), 7.88 (dd, 1H, *J* = 8.0 Hz, 2.4 Hz), 7.70 (td, 1H, *J* = 8.0 Hz, 1.2 Hz), 7.61 (td, 1H, *J* = 8.0 Hz, 1.2 Hz), 7.58-7.53 (m, 2H), 7.09 (dd, 1H, *J* = 8.0 Hz, 2.4 Hz), 6.66 (d, 1H, *J* = 2.0 Hz), 6.46 (dd, 1H, *J* = 8.0 Hz, 2.0 Hz), 6.00 (s, 1H), 4.34 (bs, 2H), 3.34 (m, 4H, *J* = 6.8 Hz), 1.16 (t, 6H, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 166.27, 153.64, 152.31, 149.36, 149.24, 141.24, 133.55, 130.78, 129.46, 128.79, 127.59, 127.04, 126.26, 125.46, 124.76, 123.26, 123.00，122.31, 114.20, 109.26, 105.27, 104.63, 98.51, 66.32, 44.61, 13.45; Anal. calcd for $C_{28}H_{23}NO_4 \cdot H_2O$; C, 71.62; H, 5.80; N, 8.95; Found: C, 71.55; H, 5.66; N, 8.67%.

L929 cell imaging using the dye 1

L929 cells were grown in RPMI 1640 supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were seeded at a density of 1 × 10^6 cells mL^{-1} for imaging on 24-well plates and allowed to adhere for 12 h. Before the experiments, the cells were incubated with the DAPI 0.1ug/ml for 12 h, then washed with phosphate-buffered saline (PBS) buffer. Subsequently, added dye **1** (15 μM) (containing 0.1% DMSO as a cosolvent) for 45 min at 37 ºC, finally washed with PBS three times. The fluorescence images were acquired through a Nikon eclipase TE2000 inverted fluorescence microscopy equipped with a cooled CCD camera.

Acknowledgements

This work was sponsored by the NNSFC (21272172, 51373122), NCET-09-0894, the NSFT (12JCZDJC21000) and the State Key Lab. Elemental-Organic Chemistry at Nankai University (NO. 1103).

Notes and references

[a] *Tianjin Key Laboratory for Photoelectric Materials and Devices, School of Material Science and Engineering, Tianjin University of Technology, Tianjin 300384, China. Fax: (+86)22-60215226; Tel: (+86)22-60216748; E-mail: xshzeng@tjut.edu.cn*

[b] *School of Materials Science and Engineering, Institute of Information Functional Materials& Devices, Harbin Institute of Technology, Harbin 150001, China.*

† Electronic Supplementary Information (ESI) available: Experimental details; spectra or other electronic format. See DOI: 10.1039/b000000x/

1 (a) Li, X.; Gao, X.; Shi, W.; Ma, H. *Chem. Rev.* 2014, **114**, 590. (b) Sameiro, M.; Goncalves, T. *Chem. Rev.* 2009, **109**, 190. (c) Chen, X.; Pradhan, T.; Wang, F.; Kim, J. S.; Yoon, J. *Chem. Rev.* 2012, **112**, 1910.

2 Chen, X.; Tian, X.; Shin, I.; Yoon, J. *Chem. Soc. Rev.* 2011, **40**, 4783.

3 Song, L.; Varma, C. A. G. O.; Verhoeven, J. W.; Tanke, H. *Biophys. J.* 1996, **70**, 2959.

4 Sjoback, R.; Nygern, J.; Kubista, M. *Spectrochim. Acta Part A* 1995, **51**, L7.

5 (a) Chapple, M. R.; Johnson, G. D.; Davidson, R. S. *J. Microsc.* 1990, **159**, 254. (b) Chen, R. F.; Knutson, J. R. *Anal. Biochem.* 1988, **172**, 61.

6 (a) Valdes-Aguilera, O.; Neckers, D. C. *Acc. Chem. Res.* 1989, **22**, 171. (b) Faraggi, M.; Peretz, P.; Rosenthal, I.; Weinraub, D. *Chem. Phys. Lett.* 1984, **103**, 310.

7 Bujdak, J.; Iyi, N. *J. Phys. Chem. B* 2006, **110**, 2180.

8 Sasai, R.; Iyi, N.; Fujita, T.; Arbeloa, F. L.; Martinez, V. M.; Takagi, K.; Itoh, H. *Langmuir* 2004, **20**, 4715.

9 (a) Lee, L. G.; Berry, G. M.; Chen, C.-H. *Cytometry*, 1989, **10**, 151. (b) Whitaker, J. E.; Haugland, R. P.; Ryan, D.; Hewitt, P. C.; Haugland, R. P.; Prendergasty, F. G. *Anal. Biochem*. 1992, **207**, 267. (c) Peng T.; Yang, D. *Org. Lett.* 2010, **12**, 496; (d) S. Kamino, H. Ichikawa, S. Wada, Y. Horio, Y. Usami, T. Yamaguchi, T. Koda, A. Harada, K. Shimanuki, M. Arimoto, M. Doi and Y. Fujita, *Bioorg. Med. Chem. Lett.* 2008, **18**, 4380.

10 (a) Dickinson, B. C.; Huynh, C.; Chang, C. J. *J. Am. Chem. Soc.* 2010, **132**, 5906. (b) Dickinson, B. C.; Chang, C. J. *J. Am. Chem. Soc.*, 2008, **130**, 9638. (c) Dickinson, B. C.; Tang, Y.; Chang, Z.; Chang, C. J. *Chem. Biol.*, 2012, **18**, 943.

11 Peng, T.; Yang, D. *Org. Lett.* 2010, *12*, 4932.

12 Kawai, K.; Ieda, N.; Aizawa, K.; Suzuki, T.; Miyata, N.; Nakagawa, H. *J. Am. Chem. Soc.* 2013, **135**, 12690.

13 (a) Kamiya, M.; Asanuma, D.; Kuranaga, E.; Takeishi, A.; Sakabe, M.; Miura, M.; Nagano, T.; Urano, Y. *J. Am. Chem. Soc.* 2011, **133**, 12960. (b) Ichikawa, Y.; Kamiya, M.; Obata, F.; Miura, M.; Terai, T.; Komatsu, T.; Ueno, T.; Hanaoka, K.; Nagano, T.; Urano, Y. *Angew. Chem. Int. Ed.* 2014, **53**, 6772. (c) Komatsu, H.; Shindo, Y.; Oka, K.; Hill, J. P.; Ariga, K. *Angew. Chem. Int. Ed.* 2014, **53**, 3993. (d) Paritala, H.; Carroll, K. S. *Anal. Biochem.* 2013, **440**, 32.

14 (a) Dong, W.; Wen, H.; Yang, X.-F.; Li, H. *Dyes Pigment.* 2013, **96**, 653. (b) Yang, X.-F.; Huang, Q.; Zhong, Y.; Li, Z.; Li, H.; Lowry, M.; Escobedo, J. O.; Strongin, R. M. *Chem. Sci*., 2014, **5**, 2177. (c) Bhuniya, S.; Maiti, S.; Kim, E.-J.; Lee, H.; Sessler, J. L.; Hong, K. S.; Kim, J. S. *Angew. Chem. Int. Ed.* 2014, **53**, 4469.

15 (a) Clark, M. A.; Duffy, K.; Tibrewala, J.; Lippard, S. J. *Org. Lett.* 2003, **5**, 2051. (b) Tomat, E.; Lippard, S. J. *Inorg. Chem*. 2010, **49**, 9113. (c) Burdette, S. C.; Lippard, S. J. *Inorg. Chem*. 2002, **41**, 6816. (c) Wen, H.; Huang, Q.; Yang, X.-F.; Li, H. *Chem. Commun.* 2013, *49*, 4956. (d) Im, H. G.; Kim, H. Y.; Chang, S.-K. *Sens. Actuators, B* 2014, **191**, 854. (e) Taki, M.; Akaoka, K.; Mitsui, K.; Yamamoto, Y. *Org. Biomol. Chem.* 2014, **12**, 4999.

16 Lv, X.; Liu, J.; Liu, Y.; Zhao, Y.; Chen, M.; Wang, P.; Guo, W. *Sens. Actuators, B* 2011, **158**, 405

17 (a) Xuan, W.; Cao, Y.; Zhou, J.; Wang, W. *Chem. Commun.* 2013, **49**, 10474. (b) Zhu, D.; Xue, L.; Li, G.; Che, Y.; Jiang, H. *Org. Chem. Front.* 2014, **1**, 501.

18 (a) Whitaker, J. E.; Haugland, R. P.; Prendergast, F. G. *Anal. Biochem.* 1991, **194**, 330. (b) Nakata, E.; Yukimachi, Y.; Nazumi, Y.; Uto, Y.; Maezawa, H.; Hashimoto, T.; Okamoto, Y.; Hori, H. *Chem. Commun.* 2010, **46**, 3526. (c) Fu, Y.; Collinson, M. M.; Higgins, D. A. *J. Am. Chem. Soc.* 2004, **126**, 13838.

19 C. Reichardt, *Solvents and solvent effects in organic chemistry*, 4th, ed., Wiley-VCH, Weinheim, 2010.

20 P. Du and S. J. Lippard, *Inorg. Chem*. 2010, **49**, 10753.

21 Mercury Update: Impact on Fish Advisories: EPA Fact Sheet EPA-823-F-01-011; EPA, Office of Water: Washington, DC, 2001: The maximum contamination concentration of mercury in drinking water is 2 ppb (10 nM).

22 (*a*) A. Coskun and E. U. Akkaya, *J. Am. Chem. Soc.* 2006, **128**, 14474; (b) W. Shi, S. Sun, X. Li and H. Ma, *Inorg. Chem*. 2010, **49**, 1206; (c) K. Rurack, M. Kollmannsberger, U. Resch-Genger and J. Daub, *J. Am. Chem. Soc.* 2000, **122**, 968; (d) Li, Y.; He, S.; Lu, Y.; Zhao, L.; Zeng, X. *Dyes and Pigments*, 2013, **96**, 424; (e) Li, Y.; He, S.; Lu, Y.; Zeng, X. *Org. Biomol. Chem*. 2011, **9**, 2606.

Organic & Biomolecular Chemistry Accepted Manuscript

This journal is © The Royal Society of Chemistry [year]