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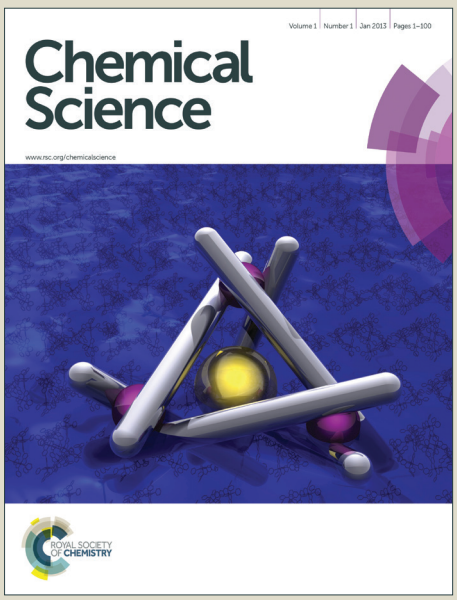

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EDGE ARTICLE

A Ratiometric Fluorescent Molecular Probe with Enhanced Two-photon Response upon Zn^{2+} Binding for *in vitro* and *in vivo* Bioimaging

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A bipyridine centered donor-acceptor-donor (D-π-A-π-D) type ratiometric fluorescent molecular probe exhibited an unprecedented enhancement in two-photon absorption (2PA) cross section upon Zn^{2+} binding. Moreover, owing to the excited state charge-transfer of the fluorophore π-backbone, a significant enhancement in the two-photon excited (λ_{exc} = 820 nm) fluorescence intensity was observed upon Zn^{2+} binding resulting in a 13-fold enhancement in the 2PA cross section and 9-fold enhancement in fluorescence brightness at 620 nm when compared to the cation free fluorophore. The high 2PA cross section of 1433 GM and 2P action cross section (860 GM) with excellent 2P excited fluorescence variation at 620 and 517 nm upon Zn^{2+}binding facilitated a ratiometric monitoring of free zinc ions in cells. The low cytotoxicity and good photostability of the fluorophore allowed two-photon Zn^{2+} imaging of HeLa cells. In addition, the *in vivo* two-photon imaging of Zn^{2+} ions in hepatocytes of live mice illustrates the viability of the probe in tissue imaging and monitoring of free zinc ions in live cells.

Introduction

Organic molecules with large two-photon absorption (2PA) cross section are of significant importance due to wide-ranging application in fluorescence microscopic imaging,[1] photodynamic therapy,[2] optical power limiting,[3] microfabrication[4] and 3D optical data storage.[5] Strategies to develop organic molecules with large 2PA include the design of dipolar, quadrupolar and octupolar structural motifs, increasing conjugation length and attaching strong electron donors/acceptor groups to the chromophore backbone.[6] Magnitude of 2PA cross section is found to be high for molecules with large excited state intramolecular charge transfer (ICT).[7] Molecular structures with an acceptor moiety placed in between two electron donors connected by a π-bond (D-π-A-π-D) exhibit large TPA cross section values when compared to the corresponding A-π-D-π-A configurations.[6b,8] D-π-A-π-D type molecules with prominent brightness upon two-photon (2P) excitation are highly useful for multi-photon imaging of biological specimen. When compared to one photon microscopy, multi-photon based approach involves the usage of low energy photons for excitation, which significantly reduces tissue auto-fluorescence, self-absorption, photodamage/photo-bleaching etc. This method also has the advantage of deeper penetration depth and localized excitation.

Two-photon active Zn^{2+} selective fluorophores are of particular interest due to the vital role of zinc in biological functions, such as gene expression, apoptosis, enzyme regulation, and neurotransmission.[9] Variation of zinc ion levels is reported to be linked with neurological disorders such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, and epilepsy.[10] The scope for the exploration of the diverse physiological roles of Zn^{2+} demands sensitive and noninvasive techniques for its real-time detection and imaging. However, estimation of spectroscopically silent free Zn^{2+} is difficult with classical methods. Therefore, design of selective and efficient fluorescent probes that target imaging of zinc ions has been of top priority to chemists. Fluorescent probes in general lack sufficient penetration depth while imaging biological tissues. Therefore, 2P active fluorescent probes with high 2PA cross-section, selective for Zn^{2+} are of great demand. However, many of the 2P active probes, especially D-π-A-π-D type molecules exhibit low 2PA values under aqueous conditions due to strong solvent-solute interaction of the polarized excited state.[11] Hence, the challenge is to design a ratiometric Zn^{2+} selective 2P active probe that exhibits high absorption cross section, enhanced fluorescence intensity upon complexation with the metal ion, good pH and photostability and better tissue penetration depth.

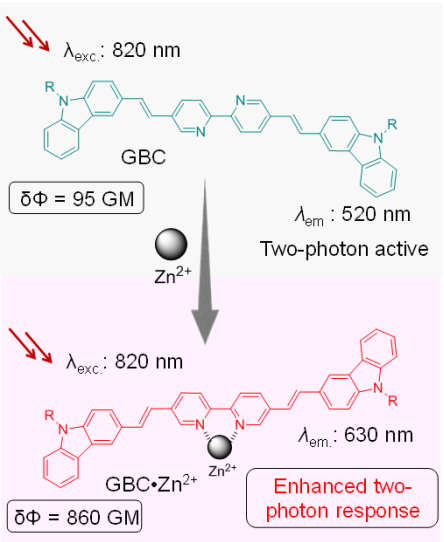

Fig. 1 Schematic representation of two-photon response of GBC and GBC•Zn^{2+}

There are several reports on 2P active fluorophores for the sensing and imaging of Zn^{2+}.[1d,12-14] However, most of them show nonratiometric fluorescence response and their 2PA cross section (δ) and 2P action cross section (δΦ) values are relatively low (Table S1†). Earlier, we have reported D-π-A-π-D type Zn^{2+} selective fluorescent probes having good fluorescence quantum yields and excited state charge transfer properties.[15] Since D-π-A-π-D type fluorophores with a central electron deficient moiety are known to exhibit strong 2PA cross section, we hypothesized that in such systems, the 2P properties may further improve upon binding with a suitable cation. As a proof-of-principle to this hypothesis, herein, we describe a new Zn^{2+} selective ratiometric 2P active molecular probe GBC with high fluorescence quantum yield (Φ), and several fold enhancement in δ and δΦ values upon Zn^{2+} binding (Fig. 1). Moreover, the presence of oxyethylene side chains in GBC facilitates aqueous compatibility and cell permeability for the imaging of free Zn^{2+} ions in live cells.

Results and discussion

Synthesis of GBC is accomplished by the end-functionalization of a bipyridine (acceptor) with a carbazole (donor) moiety, through vinylic linkages at the 5,5' position of the bipyridine using a previously reported standard procedure.[16] Horner–Wadsworth–Emmons reaction[17] of the carbazole-3-carbaldehyde with 5,5'-diphosphonate ester of 2,2'-bipyridine gave GBC in 45% yield. The product was characterized by NMR and FAB-MS analyses. The absorption spectra of GBC in different solvents are shown in Fig. S1†. In a 1:1 acetonitrile/water (HEPES, pH 7.2), the absorption spectrum exhibited a maximum at 403 nm, which showed negligible change with solvent polarity. However, the emission spectrum of GBC was sensitive to the solvent polarity (Fig. S2a†). GBC exhibited a bathochromic shift (50 nm) of the emission in different solvents in the order chloroform<acetonitrile<1:1 acetonitrile/water (HEPES, pH 7.2). The emission in chloroform showed a maximum at 480 nm, which was shifted to 505 nm in acetonitrile and was further shifted to 530 nm in 1:1 acetonitrile/water (HEPES, pH 7.2). The emission quantum yield of GBC in acetonitrile was found to be 89% (quinine sulphate Φ_f = 0.54 in 1 N H_2SO_4 as the standard). The broad emission of GBC in polar solvents such as acetonitrile and acetonitrile/water (HEPES, pH 7.2) is due to the emission from the charge transfer (CT) state, which is stabilized by polar solvents. These observations indicate the strong excited state CT associated with GBC. The time resolved fluorescence measurements of GBC showed a gradual increase in the lifetime from 1.20 ns in chloroform to 1.77 ns in acetonitrile/water (HEPES, pH 7.2) with monoexponential decay profiles (Fig. S2b†).

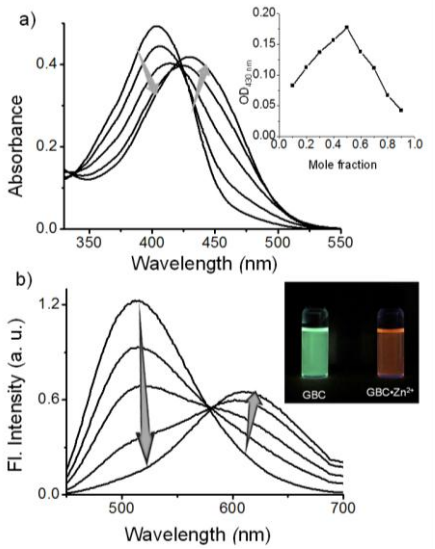

Fig. 2 Changes in the a) absorption and b) emission spectra of GBC (6μM) with the addition of $Zn(ClO_4)_2$ (0-6 μM) in 1:1 acetonitrile/water (HEPES, pH 7.2, $\lambda_{ex.}$ at 422 nm). Inset in Fig. a) shows the Job's plot showing 1:1 binding of GBC to $Zn(ClO_4)_2$ in acetonitrile/water (1:1 v/v, HEPES buffer, pH 7.2). Inset in Fig. b) shows the fluorescence colour change of GBC in the absence and presence of Zn^{2+}.

Titration of GBC with different alkali and alkaline earth metal salts showed negligible changes in the linear (one-photon) photophysical properties, whereas transition metal ions such as Zn^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+}, Pb^{2+}, Pd^{2+}, and Co^{2+} showed changes in both absorption and emission responses as exemplified with $Zn(ClO_4)_2$ in Fig. 2. Upon increasing the concentration of Zn^{2+}, GBC showed a decrease in the original absorption band at 403 nm with a concomitant formation of a new red-shifted band 430 nm as shown in Fig. 2a. Further, the reversibility of zinc complexation with GBC was confirmed with EDTA titration (Fig. S3†). Spectrochemical titration of a solution of GBC in 1:1 acetonitrile/water (HEPES, pH 7.2) with $Zn(ClO_4)_2$ showed a 1:1 stoichiometry for the complexation of Zn^{2+}, which was further confirmed by Job's plot analysis (Fig. 2a inset). Addition of transition metal salts showed quenching in the emission band except for Zn^{2+} (Fig. S4†). In the case of Zn^{2+}, a red-shifted emission band is formed followed by the quenching of the initial emission at 530 nm. Fig. 2b shows the

changes in the emission spectra of GBC (conc. 6 × 10^{-6} M, in 1:1 acetonitrile/water (HEPES, pH 7.2) when excited at 422 nm with increasing concentration of $Zn(ClO_4)_2$. Evidently, addition of $Zn(ClO_4)_2$ to a solution of GBC resulted in a new red-shifted emission band at 610 nm with a quantum yield of 60% (rhodamine B as standard Φ_f = 0.7 in ethanol). The summary of photophysical properties of GBC and GBC•Zn^{2+} is given in Table1.

Table 1 Summary of photophysical properties of GBC and GBC•Zn^{2+}

	λ_{abs} (nm)[a]	λ_{ems} (nm)[a]	Φ_f[a]	τ[d] (ns)
GBC	403	530	0.89[b]	1.77
GBC•Zn^{2+}	430	610	0.60[c]	2.30

[a]1:1 Acetonitrile/water (HEPES, pH 7.2). [b]Fluorescence quantum yield measured using quinine sulfate as standard (Φ_f = 0.54 in 1 N H_2SO_4). [c]Fluorescence quantum yield measured using rhodamine B as standard (Φ_f = 0.7 in ethanol). [d](1:1) Acetonitrile/water (HEPES, pH 7.2). Fluorescence decay profiles were recorded (± 5% error) by excitation at 375 nm, emission monitored at the emission maximum.

Since planarization induced extended π-conjugation upon zinc ion complexation generally enhances non-linear optical properties, we extended our study to investigate the two-photon absorption properties of GBC and GBC•Zn^{2+} in detail. We carried out two-photon excitation studies at 760-860 nm region using a mode locked femtosecond Ti: sapphire laser and calculated the 2PA cross section values (Fig. 3). Changes in the 2P action cross section ($\delta\Phi$) of GBC in the presence and absence of Zn^{2+} are plotted in Fig. 3a. Fluorescence of GBC and GBC•Zn^{2+} solutions upon excitation with a laser source at 820 nm is shown in Fig. 3b and 3c respectively. The two-photon absorption cross section (δ) of GBC at 800 nm was found to be 107 GM. However, Zn^{2+} complexation enhanced the 2PA cross section significantly to 1433 GM at 815 nm when compared to that of GBC alone. The observed value of δ for GBC in presence of Zn^{2+} is one of the highest ever reported for a Zn^{2+} responsive fluorophore (Table S1†). The two-photon excited fluorescence brightness is increased from 95 to 860 GM, nearly 9 times more upon binding with Zn^{2+}. The fluorescence spectrum of GBC•Zn^{2+} measured by exciting at 820 nm in presence of 6 × 10^{-5} M $Zn(ClO_4)_2$ showed ca. two-fold enhancement in the emission intensity when compared to that of GBC alone, indicating a prominent 2PA process upon Zn^{2+} complexation. 2P active chromophores with such large values of 2PA cross section and 2P excited fluorescence brightness, in response to Zn^{2+} binding has not been reported previously. It must be noted that the previous reports on large enhancement in δ and $\delta\Phi$ values are related to different chromophores and the absolute values are relatively low.[1d,12,13] Therefore, the fluorescence brightness ($\delta\Phi$) of 860 GM obtained for GBC•Zn^{2+}complex is the highest value when compared to that of other reported Zn^{2+} specific 2P probes.

Further, by a gradual increase of Zn^{2+} concentration, we monitored zinc ion induced emission changes upon two-photon excitation (800 nm) of GBC. The decrease in the fluorescence intensity of GBC at 517 nm with a concomitant increase at 620 nm corresponding to GBC•Zn^{2+} allow ratiometric monitoring of 2P excited emission response with Zn^{2+} (Fig. 3d). The ratiometric 2P excited fluorescence response I_{620}/I_{517} against the concentration of Zn^{2+} is shown in Fig. 3e. The apparent dissociation constant (K_d^{TP}) of GBC was estimated as 10 μM (Fig. 3e) with a detection limit in micromolar range. The summary of non-linear properties of GBC before and after Zn^{2+} addition is shown in Table 2.

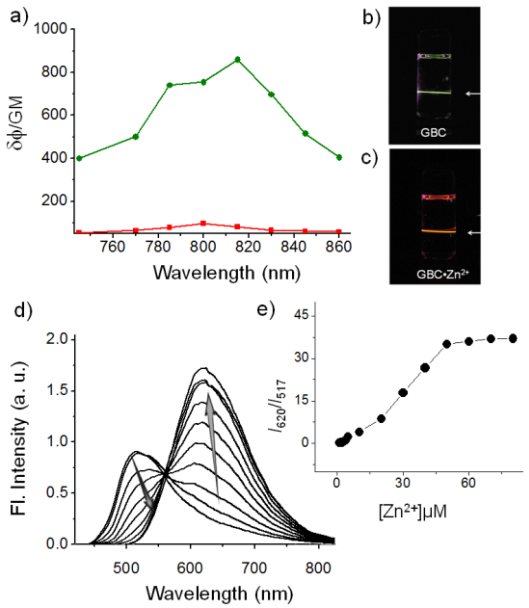

Fig. 3 2P response of GBC and GBC•Zn^{2+}. a) two-photon action spectra of GBC in the absence and presence of Zn^{2+}. Photograph showing 2P excited fluorescence from GBC in the absence (b) and presence (c) of Zn^{2+} (λ_{ex} = 800 nm). d) Two-photon excited (λ_{ex} = 800nm) ratiometric emission changes of GBC (conc. 6 × 10^{-5} M) with the addition of $Zn(ClO_4)_2$ (0-6 × 10^{-5} M) in acetonitrile/water (1:1) solution at pH 7.2. e) The ratiometric plot showing two-photon emission response of GBC with [Zn^{2+}].

Table 2 Summary of 2P properties of GBC and GBC•Zn^{2+}

	λ_{exc} (nm)[a]	δ[a, b] (GM)	$\delta\Phi$ (GM)
GBC	800	107	95
GBC•Zn^{2+}	815	1433	860

[a]1:1 acetonitrile/water (HEPES buffer at pH 7.2), [b]Conc. 6 × 10^{-5} M [1 GM = 10^{-50} cm^4s/photon].

Prior to biological studies, we investigated the photostability of GBC and GBC•Zn^{2+} in acetonitrile/water (1:1) HEPES buffer at pH 7.2 upon excitation with 808 nm laser light and monitored the changes in the absorbance at 410 nm. Reasonable photostability was observed for GBC and GBC•Zn^{2+} upon irradiation up to 2 h (Fig. S6†), which is sufficient for multiphoton imaging experiments. Further, to examine the use of GBC for bio-imaging, the inherent cytotoxicity was evaluated using 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays in HeLa (human cervix adenocarcinoma) cell lines (Fig. S7†). GBC exhibits cell viability with low to moderate toxicity even at relatively high concentration ranges.

The large 2PA cross section (δΦ) which is a measure of the 2P excited fluorescence intensity in the red region of visible spectrum is of advantage for GBC for the imaging of zinc ions in biological specimens. In order to study the two-photon *in vitro* imaging capability of GBC, HeLa cells were incubated with GBC (5μM) for 6 h following standard reported procedures. Fig. 4a-c shows 2P microscopy images recorded with 820 nm excitation and 500-600 nm emission. The fluorescence images revealed cellular uptake of GBC with good intracellular distribution. Further, GBC was incubated to another set of HeLa cell cultures pre-incubated with Zn^{2+} ions for 1 h and was imaged using the same excitation wavelength (λ_{ex} = 820 nm, emission collected: 600-700 nm). Fig. 4d-f shows the corresponding bright field, fluorescence and overlaid images respectively. The red fluorescence corresponding to GBC•Zn^{2+} clearly demonstrates the potential of GBC to image zinc ions *in vitro*.

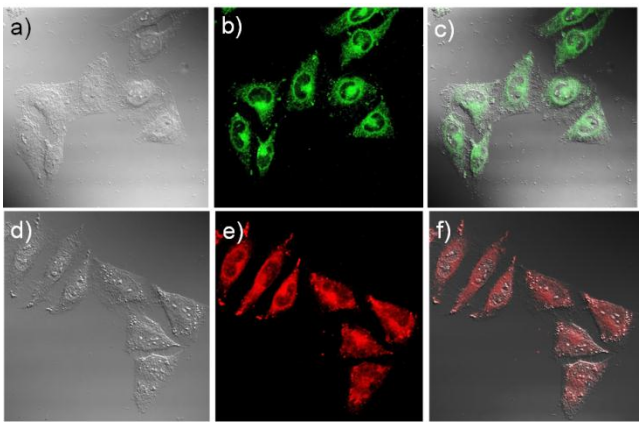

Fig. 4 2P microscopy images of HeLa cells incubated with GBC and GBC•Zn^{2+}. a) Bright field, b) fluorescence and c) overlay images of a and b of GBC alone. d) Bright field, e) fluorescence and f) overlay of images d and e obtained by *in vitro* Zn^{2+} ion monitoring using GBC in cells pre-incubated with zinc ions.

In vivo two-photon imaging of zinc ions were carried out in hepatocytes of live rat using multi-photon microscope equipped with a live animal chamber. For animal imaging, a severe combined immune deficiency (SCID) female mouse was used. The details of surgical incision and imaging techniques of hepatocytes (liver tissues) are described in the experimental section. The obtained images were processed using a Zeiss LSM image browser. Fig. 5 shows the *in vivo* 2P microscopy images of live rat hepatocytes after incubation with GBC (green emission, λ_{ex} = 820 nm, λ_{em} collected = 560-700 nm) and GBC•Zn^{2+}. Fig. 5a shows the 2P excited fluorescence image corresponding to GBC localized in the mice liver after 10 minutes of injection. Similarly, after incubation, GBC•Zn^{2+} complex shows deep red fluorescence indicating the localization inside hepatocytes (Fig. 5b). A multispectral scanning was also carried out by monitoring the emission in the wavelength window of 500-600 nm for GBC (Fig. 5c) and 560-700 nm for GBC•Zn^{2+} (Fig. 5d).

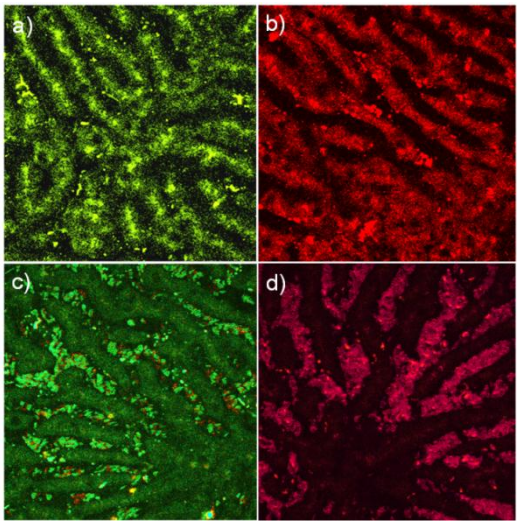

Fig. 5 *In vivo* 2P imaging using GBC and GBC•Zn^{2+}. 2P tissue image of hepatocytes in live rat after intravenous injection with a) GBC (λ_{ex} = 820 nm, λ_{em} = 530 nm) and b) GBC•Zn^{2+} (λ_{ex} = 820 nm, λ_{em} = 650 nm). Corresponding multispectral images of tissues after intravenous injection with c) GBC (λ_{ex} = 820 nm, λ_{em} = 500-600 nm) and d) GBC•Zn^{2+} (λ_{em} = 600-700 nm).

Conclusions

In conclusion, a successful design of a carbazole based D-π-A-π-D type two-photon active ratiometric probe GBC, with high 2P action cross section has been accomplished. GBC is compatible under aqueous conditions and shows selective fluorescence response to zinc ions and had sufficient photostability. In the presence of Zn^{2+}, the probe exhibited a 13-fold enhancement in the two photon absorption cross section and a 9-fold enhancement in the 2P action cross section when compared to that of the free probe. The observed δ and δΦ values are the highest ever reported for a 2P active ratiometric Zn^{2+} probe. Good cell permeability, high fluorescence quantum yield, large 2PA cross section and high 2P fluorescence brightness of GBC are of advantage for the two-photon imaging of free zinc ions in live cells which may help tracking metabolic disorders associated with zinc ion variations in cells. The demonstration of two-photon tissue imaging with live hepatocytes of rat further proves the viability of GBC as a zinc ion fluorescent probe both *in vitro* and *in vivo*.

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Notes and references

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1 W. Denk, J. H. Strickler and W. W. Webb, *Science* 1990, **248**, 73-76; W. R. Zipfel, R. M. Williams and W. W. Webb, *Nat. Biotechnol*., 2003, **21**, 1369-1377; F. Helmchen and W. Denk, *Nat. Methods*, 2005, **2**, 932-940; H. M. Kim and B. R. Cho, *Acc. Chem. Res*., **2009**, *42*, 863-872.

2 W. G. Fisher, W. P. Partridge Jr., C. Dees and E. A. Watcher, *Photochem. Photobiol*., 1997, **66**, 141-155; A. P. Castano, P. Mroz and M. R Hamblin,. *Nat. Rev. Cancer* 2006, **6**, 535-545.

3 C. W. Spangler, *J. Mater. Chem*., 1999, **9**, 2013-2020.

4 C. N. LaFratta, J. T. Fourkas, T. Baldacchini and R. A. Farrer, *Angew. Chem., Int. Ed.*, 2007, **46**, 6238-6258.

5 S. Kawata and Y. Kawata, *Chem. Rev*., 2000, **100**, 1777-1788.

6 B. Reinhardt, A. L. L. Brott, S. J Clarson,. A. G. Dillard, J. C. Bhatt, R. Kannan, L. Yuan, G. S. He and P. N. Prasad, *Chem. Mater.*, 1998, **10**, 1863-1874; Q. Zheng, G. S. He and P. N. Prasad, *J. Mater. Chem*., 2005, **15**, 579-587; G. S. He, L.-S. Tan, Q. Zheng and P. N. Prasad, *Chem. Rev.*, 2008, **108**, 1245-1330; Q. Zheng, S. K. Gupta, G. S. He, L.-S. Tan and P. N. Prasad, *Adv. Funct. Mater.*, 2008, **18**, 2770-2779; M. Pawlicki, H. A. Collins, R. G. Denning and H. L. Anderson, *Angew. Chem., Int. Ed*., 2009, **48**, 3244-3266; S. B. Noh, R. H. Kim, W. J. Kim, S. Kim, K.-S. Lee, N. S. Cho, H.-K. Shim, H. E. Pudavar and P. N. Prasad, *J. Mater. Chem*., 2010, **20**, 7422-7429.

7 A. Bhaskar, G. Ramakrishna, Z. Lu, R. Twieg, J. M. Hales, D. J. Hagan, E. V. Stryland and T. J. Goodson III, *J. Am. Chem. Soc*., 2006, **128**, 11840-11849; S. Mazzucato, I. Fortunati, S. Scoloro, M. Zerbetto, C. Ferrannte, R. Signorini, D. Pedron, R. Bozio, D. Locatelli, S. Righetto, D. Roberto, R. Ugo, A. Abbotto, G. Archetti, L. Beverina and S. Ghezzi, *Phys. Chem. Chem. Phys*., 2007, **9**, 2999-3005; C. Katan, S. Tretiak. M. H. V. Werts, A. J. Bain, R. J. Marsh, N. Leonczek, N. Nicolaou, E. Badaeva, O. Mongin and M. B. Desce, *J. Phys. Chem.* B, 2007, **111**, 9468-9483; Y. Yan, B. Li, K. Liu, Z. Dong, X. Wang and S. Qian, *J. Phys. Chem.* A, 2007, **111**, 4188-4194.

8 M. Albota, D. Beljonne, J.-L. Brédas, J. E. Ehrlich, J.-Y. Fu, A. A. Heikal, S. E. Hess, T. Kogej, M. D. Levin, S. R. Marder, D. M. Maughon, J. W. Perry, H. Rockel, M. Rumi, G. Subramaniam, W. W. Webb, X.-L. Wu and C. Xu, *Science*, 1998, **281**, 1653-1656.

9 K. H. Falchuk, *Mol. Cell. Biochem*., 1998, **188**, 41-48; P. D. Zalewski, I. J. Forbes and W. H. Betts, *Biochem. J.*, 1993, **296**, 403-408.

10 A. I. Bush, W. H. Pettingel, G. Multhaup, M. D. Paradis, J. P. Vonsattel, J. F. Gusella, K. Beyreuther, C. L. Masters and R. E. Tanzi, *Science*, 1994, **265**, 1464-1467; G. A. Howell, M. G. Welch and C. J Frederickson, *Nature*, 1984, **308**, 736-738; M. P. Cuajungco and G. Lees, *J. Neurobiol. Dis*. 1997, **4**, 137-169; C. J. Frederickson, J.-H. Koh and A. I. Bush, *Nat. Neurosci.*, 2005, **6**, 449-462.

11 S. J. K. Pond, O. Tsutsumi, M. Rumi, O. Kwon, E. Zojer, J.-L. Brédas, S. R. Marder and J. W. Perry, *J. Am. Chem. Soc.*, 2004, **126**, 9291-9306; H. M. Kim, M.-Y. Jeong, H. C. Ahn, S.-J. Jeon and B. R. Cho, *J. Org. Chem.*, 2004, **69**, 5749-5751; K. D. Belfield, M. V. Bondar, A. Frazer, A. R. Morales, O. D. Kachkovsky, I. A. Mikhailov, A. E. Masunov and O. V. Przhonska, *J. Phys. Chem. B*, 2010, **114**, 9313-9321.

12 M. Taki, J. L. Wolford, T. V. O'Halloran, *J. Am. Chem. Soc.* 2004, **126**, 712-713; C. J. Chang, E. M. Nolan, J. Jaworski, K.-I. Okamoto, Y. Hayashi, M. Sheng and S. J. Lippard, *Inorg. Chem*., 2004, **43**, 6774-6779; E. M. Nolan, J. Jaworski, K.-I. Okamoto, Y. hayashi, M. Sheng and S. J. Lippard, *J. Am. Chem. Soc*., 2005, **127**, 16812-16823; S. Sumalekshmy, M. M. Henary, N. Siegel, P. V. Lawson, Y. Wu, K. Schmidt, J.-L. Brédas, J. W. Perryl and C. J. Fahrni, *J. Am. Chem. Soc*., 2007, **129**, 11888-11889; A. Bhaskar, G. Ramakrishna, R. J. Twieg and T. J. Goodson III, *J. Phys. Chem*. C, 2007, **111**, 14607-14611; F. Qian, C. Zhang, Y. Zhang, W. He, X. Gao, P. Hu and Z. Guo, *J. Am. Chem. Soc*., 2009, **131**, 1460-1468; X.-Y. Chen, J. Shi, Y.-M. Li, F.-L. Wang, X. Wu,Q.-X. Guo and L. Liu, *Org. Lett.*, 2009, **11**, 4426-4429; C. Huang, J. Qu, J. Qi, M. Yan and G. Xu, *Org. Lett*., 2011, **13**, 1462-1465; S. Sumalekshmy and C. J. Fahrni, *Chem. Mater*., 2011, **23**, 483-500; L. Xue, Z. Fang, G. Li, H. Wang and H. Jiang, *Sens. Actuators B*, 2011, **156**, 410-415; X. Meng, S. Wang, Y. Li, M. Zhu and Q. Guo, *Chem. Commun*., 2012, **48**, 4196-4198.

13 H. M. Kim, M. S. Seo, M. J. An, J. H. Hong, Y. S. Tian, J. H. Choi, O. Kwon, K. J. Lee and B. R. Cho, *Angew. Chem., Int. Ed*. 2008, **47**, 5167-5170; G. Masanta, C. S. Lim, H. J. Kim, J. H. Han, H. M. Kim, B. R. Cho, *J. Am. Chem. Soc*., 2011, **133**, 5698-5700; I. A. Danish, C. S. Lim, Y. S. Tian, J. H. Han, M. Y. Kang and B. R. Cho, *Chem. –Asian. J.*, 2011, **6**, 1234-1240; H. M. Kim, B. R. Cho, *Chem. –Asian J*. 2011, **6**, 58-69; N. Y. Baek, C. H. Heo, C. S. Lim, G. Masanta, B. R. Cho and H. M. Kim, *Chem. Commun*., 2012, **48**, 4546-4548.

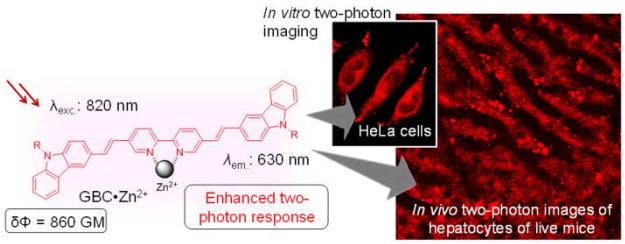
14 X.-J. Liu, J.-K. Feng, A.-M. Ren, H. Cheng and X. Zhou, *J. Chem. Phys.*, 2004, **120**, 11493-11499; S. Mazzucato, I. Fortunati, S. Scolaro, M. Zerbetto, C. Ferrante, R. Signorini, D. Pedron, R. Bozio, D. Locatelli, S. Righetto, D. Roberto, R. Ugo, A. Abbotto, G. Archetti, L. Beverina and S. Ghezzi, *Phys. Chem. Chem. Phys*., 2007, **9**, 2999-3005; C. Dragonetti, M. Balordi, A. Colombo, D. Roberto, R. Ugo, I. Fortunati, E. Garbin, C. Ferrante, R. Bozio, A. Abbotto and H. L. Bozec, *Chem. Phys. Lett.*, 2009, **475**, 245-249.

15 P. Carol, S. Sreejith and A. Ajayaghosh, *J. Am. Chem. Soc.*, 2005, **127**, 14962-14963; P. Carol, S. Sreejith and A. Ajayaghosh, *Chem. –Asian J.*, 2007, **2**, 338-348; S. Sreejith, K. P. Divya and A. Ajayaghosh, *Chem Commun.*, 2008, 2903-2905; K. P. Divya, S. Sreejith, B. Balakrishna, P. Jayamurthy, P. Anees and A. Ajayaghosh, *Chem. Commun.*, 2010, **46**, 6069-6071; S. Sreejith, K. P. Divya, T. K. Manojkumar and A. Ajayaghosh, *Chem. –Asian. J.*, 2011, **6**, 430-437; S. Sreejith, K. P. Divya, P. Jayamurthy, J. Mathew, V. N. Anupama, D. S. Philips, P. Anees and A. Ajayaghosh, *Photochem. Photobiol. Sci.*, 2012, **11**, 1715-1723.

16 J. Eldo, E. Arunkumar and A. Ajayaghosh, *Tetrahedron Lett.*, 2000, **41**, 6241-6244; A. Ajayaghosh and J. Eldo, *Org. Lett.*, 2001, **3**, 2595-2598.

17 W. S. Wadsworth and Jr. W. D. Emmons, *J. Am. Chem. Soc.*, 1961, **83**, 1733-1738.

TOC Graphics



The ratiometric two-photon (2P) probe GBC shows enhanced 2P activity upon zinc ion binding and has been used for zinc ion imaging *in vitro* and *in vivo.*