Organic & Biomolecular Chemistry

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A BODIPY-based fluorescent dye for mitochondria in living cells, with low cytotoxicity and high photostability†

Cite this: *Org. Biomol. Chem.*, 2013, **11**, 555

Received 29th September 2012,
Accepted 29th November 2012

DOI: 10.1039/c2ob26911b

www.rsc.org/obc

Si Zhang,[a] Tong Wu,[a] Jiangli Fan,[a] Zhiyong Li,[a] Na Jiang,[a] Jingyun Wang,*[b] Bairui Dou,[b] Shiguo Sun,[a] Fengling Song[a] and Xiaojun Peng*[a]

A BODIPY-based dye, OBEP, has been developed to act as a mitochondrial fluorescence probe. This dye is of high stability, low toxicity and insensitive in a pH range as wide as pH 2–10. Its uptake into mitochondria is independent of mitochondrial membrane potential in living cells. OBEP can label swollen mitochondria resulting from different degrees of cell damage in light and resist fading even after 12 h of incubation.

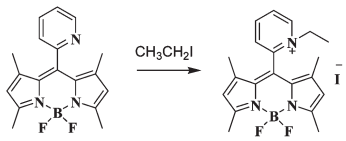

Fig. 1 Molecule structure and synthesis of **OBEP**.

Introduction

Mitochondria, the principal energy-producing compartment in most cells, have roles in a number of vital cellular processes – and as a result these organelles are crucially involved in a variety of pathologies, from Alzheimer's disease to cancer to diabetes.[1–4] Mitochondria are particularly sensitive to a variety of damaging influences.[5] When cells are injured, changes in the number, size and structure of mitochondria are often seen. Mitochondrial swelling is perhaps the most common phenomenon, with the organelles morphology changing from filamentous to round or even spheroidal forms.[5,6]

Consequently, mitochondria-staining reagents and techniques are of great importance for biomedical research[7–11] and potentially also for diagnosis. Although there is a wide variety of mitochondrially localizing fluorescent probes available commercially,[12,13] nevertheless many suffer from various practical limitations, not least of which is poor light fastness.[14–16] This being so, a significant demand remains for mitochondrially selective probes which are of high bio- and photo-stability, low cytotoxicity, and which, in order to facilitate tissue penetration, possess various long-wavelength emission peaks.

Herein we report a new fluorescent mitochondrial probe (**OBEP**, in Fig. 1) based on BODIPY. It shows excellent lightfastness, low toxicity, and has long wavelength absorption and emission (λ_{ex} 520 and λ_{em} 540 nm). As uptake of this dye is independent of mitochondrial membrane potential, it may be particularly suited to act as a mitochondrial tracker, and also to distinguish mitochondria exhibiting various degrees of swelling, and hence to indicate cell damage.

As is well known, BODIPY derivatives have been widely used as protein labeling reagents[17] and as small molecule[18] or ion sensors.[19,20] In part this reflects the high photostability of the fluorophore skeleton, and the insensitivity of its fluorescence to solvent polarity or pH.[21,22] Although BODIPY dyes suitable for imaging the endoplasmic reticulum and lysosomes have been developed commercially,[23] to our knowledge, mitochondrially localizing BODIPY derivatives are not available for purchase. Recently, Chang *et al.* used a mitochondrial-targeting triphenylphosphonium moiety for localizing the BODIPY derivative to this organelle.[20] But the toxicity is a particular concern for triphenylphosphonium.[2] **OBEP** is a cationic compound derived from quaternization of the pyridyl group in **OBP** (Fig. 1). Although BODIPYs bearing a quaternarized pyridine moiety in their *meso* position have been reported in the literature to get functional dyes suitable for various applications, their weak fluorescence limits their further development.[24–27] This precursor was a BODIPY derivative prepared from picolinaldehyde and 3,5-dimethylpyrrol *via* published procedures[24] as described in the ESI.†

[a]*State Key Laboratory of Fine Chemicals, Dalian University of Technology, 2 Linggong Road, Dalian, P.R. China. E-mail: pengxj@dlut.edu.cn; Fax: +86-411-84986306; Tel: +86-411-84986306*

[b]*A School of Life Science & Biotechnology, Dalian University of Technology, 2 Linggong Road, Dalian 116024, P.R. China. E-mail: wangjingyun@dl.cn*

†Electronic supplementary information (ESI) available: Experimental details and characterization of **OBEP**, measurement equipment and materials, measurement of partition coefficient, cell culture and staining, cell cytotoxicity assay and effects of CCCP on uptake of dyes, UV-vis absorption and fluorescence spectra of **OBP** or **OBEP** in different solvents. See DOI: 10.1039/c2ob26911b

Results and discussion

The spectroscopic properties of **OBEP** in different solvents are summarized in Table 1 and Fig. S2.† The maximum absorbance and emission wavelengths of **OBEP** were centered at 520 nm and 541 nm in acetonitrile, these peaks being almost unchanged in an aqueous buffer. Moreover, **OBEP** has a similar fluorescence quantum yield in aqueous and organic solvents ($\Phi_{F(buffer)}$ = 0.13, $\Phi_{F(acetonitrile)}$ = 0.20). Overall, this suggests that solvent polarity has only a minor influence on **OBEP** fluorescence behaviour.

As is well known, photo-bleaching is a common problem for most organic dyes, often compromising the temporal monitoring of dynamic events inside cells due to fading of the reporting dyes.[28,29] So it is significant that the photostability of **OBEP** was much higher than that of widely used dyes localizing in mitochondria of live cells, such as MitoTracker Red CMXRos and MitoTracker Green FM (Fig. 2a, after 2 h radiation, **OBEP** remains 98%, while MitoTracker Green FM is less than 5% in density).

Moreover, in a pH-titration study, the fluorescence signal of **OBEP** changed very little over the range pH 2–10, while the relative fluorescence intensity of Mitotracker Red CMXRos changed from 0.8 to 0.2 over the cellular pH 4–8 (Fig. 2b). This indicates that **OBEP** imaging would not be influenced by the cellular pH micro-environment.

To examine the imaging performance achieved using these BODIPY derivatives, HeLa cells were incubated with **OBEP** (5 μM) and **OBP** (2 μM) at 37 °C for 30 min. As shown in Fig. 3a, 3b and S4,† both dyes proved to be membrane permeant. Fluorescence was seen in the cytoplasmic but not the nuclear regions. Nevertheless, the detailed patterns of staining seen with **OBEP** and **OBP** were markedly different. **OBEP** gave rise to fluorescent filaments, which were sometimes a little swollen. On morphological grounds these were judged to be mitochondria. **OBP** was quite different, lacking filaments but instead showing a somewhat diffuse staining restricted to particular parts of the cytoplasm, plus granular cytoplasmic staining, with the nuclear periphery also taking up the dye. On morphological grounds this suggests staining of the Golgi apparatus and the nuclear membrane, the granules corresponding to either lipid droplets, lysosomes or swollen mitochondria.

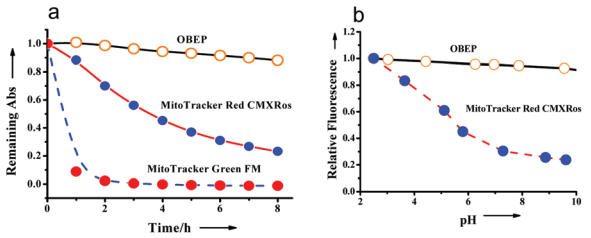

Fig. 2 (a) Comparisons of the photofading of **OBEP**, MitoTracker Red CMXRos and MitoTracker Green FM in Tris-HCl buffer (pH = 7.4); (b) influences of pH on the fluorescent emission at 541 nm of **OBEP** (5 μM) or at 602 nm of MitoTracker Red CMXRos in aqueous solution, λ_{ex} = 500 nm or λ_{ex} = 560 nm. The pH of the solution was adjusted by adding aqueous NaOH or HCl.

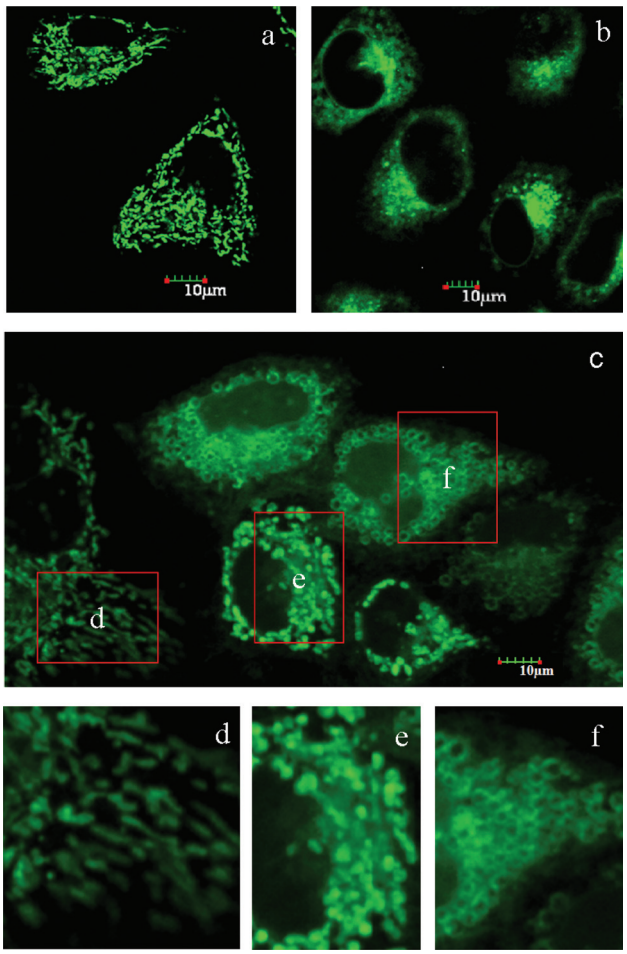

Fig. 3 Confocal fluorescence images of **OBEP** (a) or **OBP** (b) in HeLa cells. The cells were incubated with 5 μM **OBEP** or 2 μM **OBP** for 30 min; (c) various damaged mitochondrial forms. The cells were cultured with 5 μM **OBEP** for 1 h in HeLa cells; (d), (e), (f) the cells in image (c) enlarged. The images were taken under FITC channel. Scale bar = 10 μm.

Table 1 Photophysical data of **OBEP** and **OBP**

Compound (solvent)	λ_{abs}[a] (nm)	λ_{em}[b] (nm)	Stokes shift (nm)	ε_{max}[c] (M^{-1} cm^{-1})	Φ_F[d]
OBEP (buffer[e])	520	541	21	6.3×10^4	0.13
OBEP (acetonitrile)	520	542	22	6.7×10^4	0.20
OBP (buffer[e])	502	514	12	5.6×10^4	0.62
OBP (acetonitrile)	501	512	11	6.9×10^4	0.06

[a] Absorption maxima. [b] Emission maxima. [c] Molar extinction coefficient. [d] Relative quantum yield compared with fluorescein in 0.1 mol L^{-1} NaOH (Φ_F = 0.90) as the standard. [e] Tris-HCl buffer, pH = 7.4, 10 mM.

These morphological assessments are in agreement with known structure–localization relationships for dyes in live cells. **OBEP** is a quaternary salt, and so will be cationic at all cellular pHs. This dye was found to be lipophilic, with a log P = 0.9. Such lipophilic cations are known to localize in mitochondria.[30] However under neutral conditions, **OBP** will occur

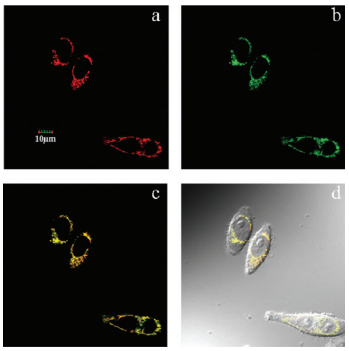

Fig. 4 Confocal images of **OBEP** plus a mitochondrially specific dye in HeLa cells. (a) Fluorescence image of HeLa cells stained with MitoTracker Deep Red FM; (b) fluorescence image of HeLa cells stained with **OBEP**; (c) merged image of (a) and (b); (d) merged image of (c) and bright field. Scale bar = 10 μm.

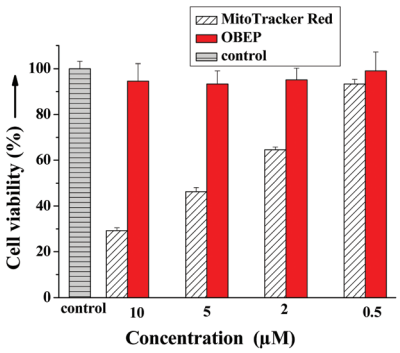

Fig. 5 Comparisons on the cytotoxicity of **OBEP** and MitoTracker Red CMXRos at various concentrations (0.5 μM, 2 μM, 5 μM, 10 μM) in living HeLa cells for 12 h.

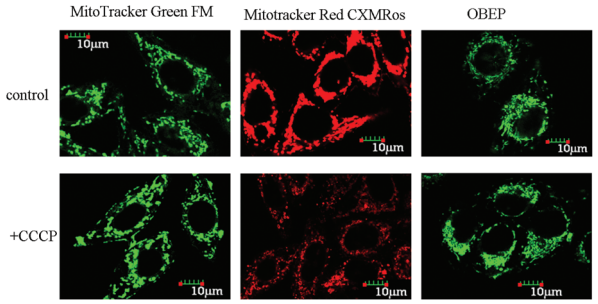

Fig. 6 Staining patterns of **OBEP** and mitochondrially specific dyes in HeLa cells. The cells were incubated in the absence or presence of 10 μM CCCP for 30 min, and then stained with 0.4 μM MitoTracker Green FM for 15 min, 0.2 μM MitoTracker Red CXMRos for 15 min, and 5 μM **OBEP** for 1 h. Scale bar = 10 μm.

as an electrically neutral free base. This species was found experimentally to be strongly lipophilic, with log P = 4.0. Such non-ionic lipophilic molecules are known to be taken into most cell membranes, including the Golgi apparatus and nuclear membrane.[31] However since **OBP** contains a weakly basic pyridyl moiety, the dye will be protonated within acid organelles such as lysosomes. This will result in its being ion-trapped in such structures.[32]

To further experimentally investigate the subcellular localization of **OBEP**, a commercially available, mitochondrially localizing dye (MitoTracker Deep Red FM) was employed for a colocalization study. As shown in Fig. 4, **OBEP** was colocalized within mitochondria. Staining experiments were also performed with another cell line, namely MCF7 (Fig. S4, S6†). The results showed that the same mitochondrial localization of **OBEP** occurred in different cell lines. With different incubation times, **OBEP** was found to localize selectively in mitochondria, with an intense fluorescent signal and a higher accumulation at 1 h than 15 minutes (Fig. S5†). A fluorescent dye localizing in the DNA of the cell nuclei (DRAQ5) was also applied in a colocalization study (Fig. S4†), no colocalization occurring in this case.

Although, as noted above, **OBEP** is relatively resistant to photodamage, nevertheless over-exposure to excitation light can give rise to mitochondrial swelling. In Fig. 3c various damaged mitochondrial forms are seen, apparently depending on the distance from the locus of an overextended excitation illumination. Thus Fig. 3d represents hollow spheres of extremely swollen mitochondria; in Fig. 3e the swelling is less developed so that most mitochondria appear as solid granules; whilst Fig. 3f contains minimally damaged mitochondria which retain a filamentous morphology.

The absence of membrane blebbing or rounding up of the cells suggested that **OBEP** was of low cell toxicity. Since this is a key feature for living cell imaging,[33] **OBEP** was compared with the commercially available and widely used mitochondrial probe MitoTracker Red CXMRos in a cytotoxicity assessment test using the MTT test. As shown in Fig. 5, the two dyes had similar ranges of cytotoxicity in low concentrations (cell viability ≥ 93% for both), but **OBEP** was less cytotoxic than MitoTracker Red CXMRos in high concentrations (such as the case of 10 μM, cell viability 94% for **OBEP** *via* 29% for MitoTracker Red CXMRos).

The staining of **OBEP** in HeLa cells treated with a membrane-potential uncoupler was examined. The uncoupler, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), can disrupt mitochondrial membrane potential (Fig. 6). Staining with MitoTracker Green FM, a mitochondrial probe whose uptake is independent of membrane potential, was the same in the presence or absence of CCCP; while staining with MitoTracker Red CXMRos, a mitochondrial probe whose uptake is membrane potential dependent, was inhibited by the presence of CCCP.[9,23,34] The staining of **OBEP** was the same in the absence or presence of CCCP, suggesting that the staining properties of **OBEP** are independent of the mitochondrial membrane potential.

Because of the excellent light-fastness and low toxicity of **OBEP**, long term fluorescence imaging experiments are possible with this dye.[29] For example, even after 12 h, the staining pattern in live MCF7 cells remained the same as those obtained by incubating for 1 h, and the fluorescence intensity remains strong (Fig. 7). These results indicate that **OBEP** is a potentially useful probe for monitoring mitochondria during isolation and following cell fusion.

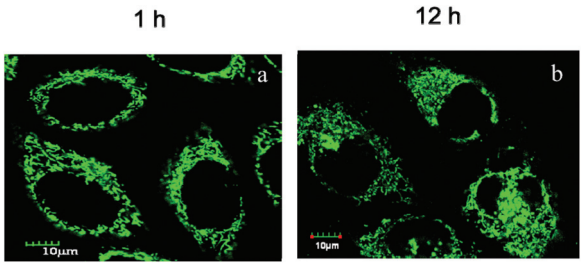

Fig. 7 Fluorescence confocal images of **OBEP** in MCF7 cells for 1 h (a) or 12 h (b). Scale bar = 10 μm.

Conclusions

In conclusion, we report a mitochondrially specific fluorescent probe, **OBEP**, which is of excellent light-fastness, low toxicity and whose uptake is independent of mitochondrial membrane potential in living cells. **OBEP** is a possible probe for following mitochondria during isolation and after cell fusion. The emission color, orange, provides additional color options for biological imaging.

Acknowledgements

This work was supported by the National Science Foundation of China (21136002, 21076032, 20923006), the National Basic Research Program of China (2009CB724706), the Ministry of Education of China (the Fundamental Research Funds for the Central Universities, DUT10LK05 and DUT10ZD114), and the Scientific Research Fund of Liaoning Provincial Education Department (LS2010040).

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