ChemComm

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Journal:	*ChemComm*
Manuscript ID:	CC-COM-06-2015-004731.R1
Article Type:	Communication
Date Submitted by the Author:	06-Jul-2015
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Journal Name

COMMUNICATION

A red emitting mitochondria-targeted AIE probe as an indicator for membrane potential and mouse sperm activity

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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In this work, a red emission AIE active mitochondrial probe is developed. It is the first non-selfquenching mitochondria specific probe with membrane potential sensitivity. Its application in sensing the membrane potential differences in mouse sperm cells is demonstrated.

Mitochondria are dynamically organelles that exist in almost all eukaryotic cells.[1] The mitochondrial morphology is regulated by a set of proteins.[1,2] The mutations of these proteins are reported to be associated with diseases, including neurodegenerative and cardiovascular diseases.[3] The major function of mitochondria is to generate energy.[4,5] Approximately, 95% of the primary source of energy used in eukaryotic cells and ATP is produced by mitochondria. In order to synthesize ATP, mitochondria continuously oxidize substrates and maintain a proton gradient across the lipid bilayer in the respiratory electron transport chain with a large membrane potential ($\Delta\Psi_m$). The $\Delta\Psi_m$ is a vital parameter reflecting the mitochondrial functional status, and thus closely related to cell health, injury and function.[2] The maintenance of their mitochondrial function is thus crucial.[6] The mitochondria $\Delta\Psi_m$ is an essential indicator for assessing the physiology, viability, and fertilization potential of sperm, the germ cell of male.[7] As the mitochondria provide energy for sperm movement, abnormal of $\Delta\Psi_m$ in sperm mitochondria may lead to mitochondria dysfunction and result in male infertility.[8] Consequently, development of efficient methods for monitoring mitochondrial morphology as well as $\Delta\Psi_m$ is of great importance for both biomedical research and early diagnosis of the related diseases.

Various cationic fluorescent dyes have been developed to target mitochondria,[9] and some of the dyes, such as Rhodamine 123 (Rh 123), TMRE/TMRM and JC-1, can be utilized to indicate mitochondria $\Delta\Psi_m$.[10] However, the photostability of these dyes leaves much to be desired due to the detrimental concentration-quenching emission effect. Such effect only allows the use of very dilute solution of the probes for imaging (generally < 1–30 nM),[11] which easily leads to fast photobleaching of the probes when a harsh laser beam is used as the excitation light source. The situation is even more complicated when the concentration-quenching dyes are used to measure the $\Delta\Psi_m$. For example the elevation of the $\Delta\Psi_m$ resulting in the increase of dye amounts in the mitochondria may lead to either the enhancement or the decrease of the fluorescent signal, depending on whether the dye concentration is within the non-quenching range. JC-1 is the most widely used fluorescent indicator for $\Delta\Psi_m$. However, JC-1 is highly sensitive to dye loading concentration and time. Many literatures reported the complexities and false results of using JC-1 for measuring $\Delta\Psi_m$.[11] Therefore, it is highly demanded to develop a non-selfquenching photostable mitochondrial probe to reveal the $\Delta\Psi_m$ in living cells.

In 2001, we discovered a unique photophysical phenomenon of aggregation-induced emission (AIE).[12] Luminonegns with AIE properties are almost nonfluorescent in the solution state but become highly emissive when aggregates are formed. Restriction of intramolecular rotation (RIR) is deemed to be the major mechanism for the AIE effect.[13] The inherent property of AIE luminogens has fuelled their application as probes for cell imaging and tracking.[14] Impressively, the photostability of these luminogens has been greatly improved. Through our molecular endeavour, we have successfully developed a couple of mitochondria-targeting AIE probes.[15,16] However, most of these probes emit at short wavelength region and are unresponsive to the $\Delta\Psi_m$ changes. On the other hand, probes emitting at longer wavelength region offer various advantages such as minimum photo-damage to biological samples, deep tissue penetration and little interference from autofluorescence.[17] Efficient red emitting probes with excellent

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Electronic Supplementary Information (ESI) available: available: Experimental details, spectral and imaging data. See DOI: 10.1039/x0xx00000x

photostability and functionality are thus highly desirable but still rare.

Fluorophores that undergo intramolecular charge transfer (ICT) based on donor-acceptor architecture are widely used as red emitters.[18] The indolium salt, which serves as an electron-accepting moiety in many ICT luminogens such as cyanine dyes, is positively charged, which is essential for mitochondrial-targeting probe.[19] We thus designed and synthesized two cationic luminogens, TPE-In and TPE-Ph-In, by incorporating indolium salt into the typical AIE unit-tetraphenylethylene (TPE) (Fig. 1). As anticipated, both of them are AIE-active and emit at the red region in their aggregate state with a large Stokes shift (>200 nm). Interestingly, TPE-Ph-In exhibits more significant AIE effect and lower cytotoxicity than TPE-In. TPE-Ph-In can specifically stain the mitochondria in living cells with superior photostability. Moreover, it exhibits the unique ability to evaluate the mitochondrial $\Delta\Psi_m$ and trace the change of in situ $\Delta\Psi_m$ in living cells and thus could be used to indicate mouse sperm activity.

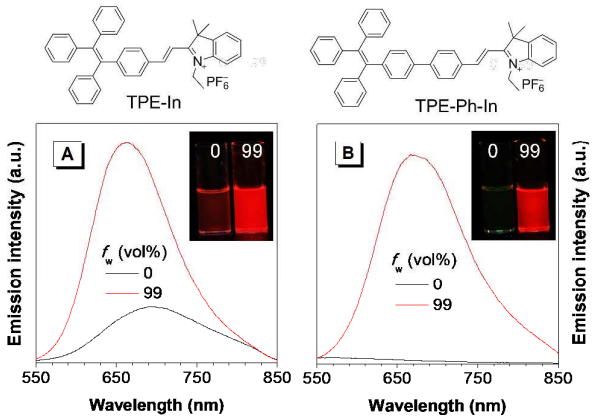

Fig. 1 Structures and emission spectra of (A) TPE-In and (B) TPE-Ph-In in DMSO and DMSO/water mixtures with 99% water fractions (f_w). Dye concentration: 10 μM; excitation wavelength: 450 nm. Inset: photographs of (A) TPE-In and (B) TPE-Ph-In in DMSO/water mixtures with f_w values of 0 and 99 vol% under 365 nm UV irradiation.

TPE-In and TPE-Ph-In were prepared by the Knoevenagel condensation of the appropriate aldehydes with indolium salt, as shown in Scheme S1. The structures were characterized by NMR and mass spectroscopy, and both gave satisfactory analysis data corresponding to their molecular structures. The absorption of TPE-In and TPE-Ph-In are peaked at ca. 445 nm in DMSO (Fig. S7). As shown in Fig. 1, TPE-In emits weakly at 694 nm in DMSO. When the water fraction reached 99% in the solvent mixture, stronger red fluorescence was observed. The AIE effect is even more obvious for TPE-Ph-In. The emission of TPE-Ph-In is enhanced about 70 times upon aggregates formation. The fluorescence quantum yields of their powders determined are approximately 11.3 and 9.7 %, respectively. Both TPE-In and TPE-Ph-In are AIE active and thus free of the self-quenching problem encountered by most of the conventional mitochondria probes, such as Rh 123 (Fig. S8).[20]

The cytotoxicity of the two luminogens on HeLa cells was assessed using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. As illustrated in Fig. S9, the cell viability is significantly reduced when 5.0 μM TPE-In is added to the HeLa cells. In contrast, TPE-Ph-In shows almost no cytotoxicity. Even when the concentration of TPE-Ph-In in the culture medium is as high as 8.0 μM, the cell growth is not obviously affected. The planarity of TPE-In may facility the molecules to interact with DNA and thus lead to higher cycotoxicity.[21] As depicted in Fig. S10, TPE-In exhibits better planarity: one of the phenyl rings on the TPE unit is coplanar with the indolium unit, whereas TPE-Ph-In adopts more twisted configuration.

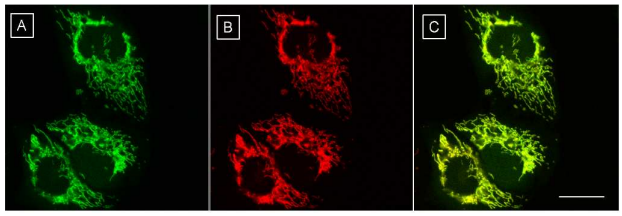

Fig.2 Confocal images of HeLa cells stained with (A) Mito-GFP, (B) TPE-Ph-In (5 μM), (C) Panels A and B merged. Scale bar: 20 μm.

TPE-Ph-In was thus chosen for cell staining test. Pre-experiment showed that this dye is cell permeable and stain mitochondria specifically. Compared with TPE-TPP, the UV-excited AIE mitochondrial probe with blue-emission previously developed in our group, TPE-Ph-In can be well excited with a 488 nm laser and therefore more compatible with confocal microscope and manifest higher signal-to-noise ratio (Fig. S11). To test the mitochondrial selectivity of TPE-Ph-In, a co-localization experiment was performed with commercial CellLight® Mitochondria-GFP (Mito-GFP), a green fluorescent protein targeted to mitochondria. The stained cells give out red fluorescence from TPE-Ph-In and green fluorescence from Mito-GFP (Fig. 2A and 2B), respectively. Merged image shows that the distribution of TPE-Ph-In in cells is totally consistent with that of Mito-GFP, indicating the high selectivity of TPE-Ph-In towards mitochondria.

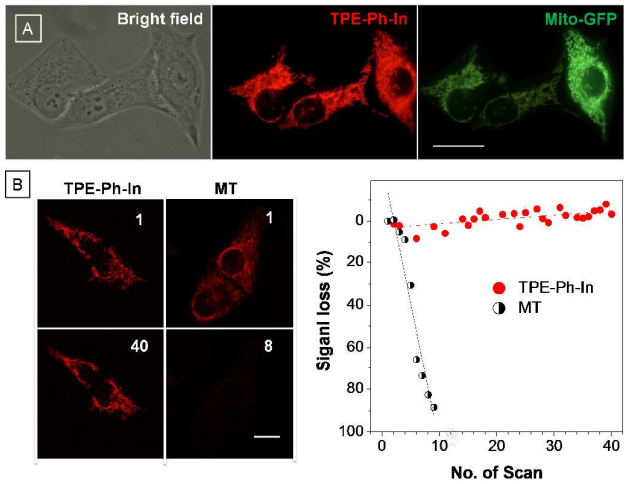

Fig.3 (A) Mito-GFP and TPE-Ph-In co-stained cells in one image field, Scale bar: 30 μm. (B) Confocal images of HeLa cells stained with TPE-Ph-In and MitoTracker Red FM (MT) taken under continuous excitation at 488 nm for 40 scans and 560 nm for 9 scans, (Scale bar: 20 μm) and the signal loss (%) of fluorescent intensity of TPE-Ph-In and MT with increasing number of scans.

The advantage of small organic dye over fluorescent protein for cell staining is also revealed in above experiment. In the co-staining experiment, we observed that all the cells are uniformly stained

with the red emitting TPE-Ph-In, while in the green channel of Mito-GFP, several cells emit obviously weaker than the others in some image field (Fig. 3A). This should be attributed to the inhomogeneous transfection rate and the different expression levels of Mito-GFP in cells, which is also mentioned in the Mito-GFP user manual.[22]

To investigate the photostability of TPE-Ph-In, the TPE-Ph-In stained cells were continuously scanned by a confocal microscope. As described in Fig. 3B, after 40 scans, the signal loss of TPE-Ph-In is less than 10% of the original intensity, and no significant difference was observed between the first and the 40th fluorescent images. MitoTracker red FM (MT), a commercially red emission mitochondria probe was tested under the same excitation power. In comparison, the fluorescence signal loss of MT reaches up to 90% of the initial value and almost no fluorescence is remained after only 8 scans. Undoubtedly, TPE-Ph-In possesses much higher photostability than commercial MT, implying its capability in long-term mitochondrial imaging and morphological analysis.

The $\Delta\Psi_m$ is the major driving force for cationic lipophilic dyes to enter and stain the mitochondria. Thus, we envisioned that we could evaluate the mitochondrial function by a $\Delta\Psi_m$ sensitive probe. To exam the response of TPE-Ph-In towards $\Delta\Psi_m$ changes, membrane-potential stimulants, oligomycin and carbonyl cyanide 3-chlorophenylhydrazone (CCCP), were applied to increase or decrease the $\Delta\Psi_m$ prior to the staining process, respectively. When the cells were treated with oligomycin, significant enhancement of the red fluorescent signals was observed (Fig. S12). Once the cells are treated with CCCP, TPE-Ph-In can no longer effectively accumulate in the mitochondria, resulting in the decrease of the fluorescent signals (Fig. S13). These results demonstrate that the accumulation of TPE-Ph-In in the mitochondria depends on the $\Delta\Psi_m$. More importantly, the fluorescent signals of TPE-Ph-In can directly represent the $\Delta\Psi_m$ based on the positive correlation between the fluorescent intensity and the local dye concentration in mitochondria, which is difficult to achieve for traditional dyes suffering from concentration quenching effect.

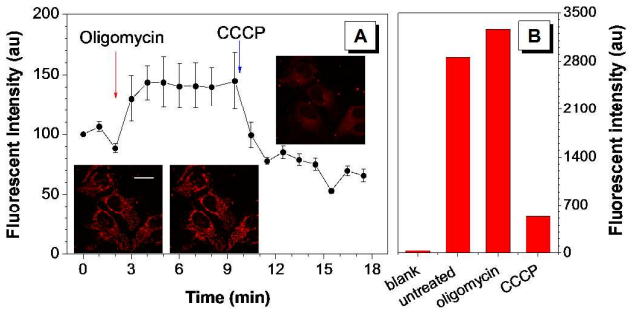

Fig. 4 (A) Changes of emission intensity of HeLa cells stained with TPE-Ph-In (5 μM) upon treated with 10 μg/mL oligomycin and then 20 μM CCCP. Excitation wavelength: 488 nm. Inset: snapshots of the cells in different period of time during the treatment of stimulants. Scale bar: 20 μm. (B) The fluorescent intensity of the unstained blank HeLa cells, untreated TPE-Ph-In stained HeLa cells, Oligomycin treated TPE-Ph-In stained HeLa cells and CCCP treated TPE-Ph-In stained HeLa cells analysed by flow cytometry.

To further examine the feasibility of using TPE-Ph-In to follow the real-time in situ change of $\Delta\Psi_m$ in living cells, we added different stimulants sequentially and collected the fluorescent intensity and imaging by confocal microscope. As shown in Fig. 4A, HeLa cells were first stained with 5 μM TPE-Ph-In for 30 min. The red emission from mitochondria was observed. The fluorescence signal was increased by 1.5-fold when the cells were treated with 10 μg/mL oligomycin. The enhanced emission from mitochondria can be retained for a period of time. Further addition of 20 μM CCCP into the medium immediately leads to drastic decrease of emission intensity. As mentioned above, the treatment of oligomycin and CCCP can lead to the increase and decrease of $\Delta\Psi_m$, respectively, which is reflected on the change of the fluorescence signal of TPE-Ph-In. Because of its high signal-to-noise ratio and low background, no washing step is required during the entire process, thus providing a convenient method for tracing the micro-environment changes in living cells.

In order to explore the application of TPE-Ph-In for high-throughput analysis, we also adopted flow cytometry in the study. As shown in Fig. S14B, the HeLa cells stained with 4 μM TPE-Ph-In for 30 min exhibit the mean fluorescent intensity about 2866, which can be easily differentiated from the unstained cells (Fig. S14A). In accordance with the results of the confocal images, a slightly increase of the mean fluorescent intensity was detected when the cells were treated with oligomycin for 25 min (Fig. S14C). In sharp contrast, after incubation of TPE-Ph-In stained cells and CCCP for 25 min, the mean fluorescent intensity was decreased for 5.4-fold (Fig. S14D), which is also consistent with the result collected by confocal microscopy. The mean intensity is plotted in Fig 4B. The use of flow cytometry thus offers a high-throughput and quantitative analysis manner for TPE-Ph-In to probe cells in different environment.

The good biocompatibility and the membrane potential-dependent fashion of TPE-Ph-In inspired us to explore its feasibility to evaluate the sperm vitality. Mouse sperm cells were stained with 5 μM TPE-Ph-In for 1 h. Under the fluorescent microscope, the midpieces of sperms present various degree of fluorescence intensity, as illustrated in Fig. 5. To gain further insight into this phenomenon, the dynamic motion of sperms stained with TPE-Ph-In was tracked and recorded (Video 1). The result reveals the bright red fluorescence comes from the energetic sperms, while the unvital sperms only give faint red fluorescence and even nonfluourescence. The fluorescent intensity in the TPE-Ph-In stained mitochondria well reflects the mitochondrial mobility, suggesting that TPE-Ph-In is a promising fluorescent probe for monitoring the function of sperm.

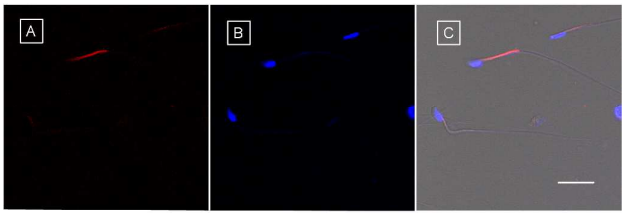

Fig. 5 Confocal images of mouse sperm cells stained with (A) TPE-Ph-In (5 μM) for 1 h and (B) Hoechst 33342 (1 ug/mL) for 10 min; and (C) the merged picture of A, B and the bright field image. Excitation wavelength: 488 nm (for TPE-Ph-In) and 405 nm (for Hoechst 33342). Scale bar: 20 μm.

In conclusion, we have designed and synthesized two AIE-active luminogens TPE-In and TPE-Ph-In emitting at the red region. Compared with TPE-In, the presence of an extra phenyl ring in TPE-Ph-In endows it with more significant AIE effect and better

biocompatibility. Due to its appropriate cationic and lipophilic features, TPE-Ph-In displays prominent specificity to mitochondria in living cells with sensitivity towards mitochondrial $\Delta\Psi_m$. It possesses excellent photostability owing to the AIE property. The morphology of mitochondria can be well presented and monitored with the assistant of TPE-Ph-In. Without the interference of the concentration-quenching problem, the fluorescent signal of TPE-Ph-In can directly represent the difference in $\Delta\Psi_m$. We thus applied TPE-Ph-In for monitoring the change of $\Delta\Psi_m$ in situ by using confocal microscopy and flow cytomerty, demonstrating its great potential for probing and tracing the change of intracellular $\Delta\Psi_m$. The evaluation of the sperm vitality is also achieved. Moreover, this probe could be used for indicating the elevated $\Delta\Psi_m$ in tumor cells for cancer research or the decreased $\Delta\Psi_m$ in apoptotic cells for drug screening.

We thank Prof. H. Benjamin Peng and Mr William K. W. Chau in HKUST for their helpful suggestions and technical support. This work was partially supported by National Basic Research Program of China (973 Program; 2013CB834701), the Research Grants Council of Hong Kong (604913, 16301614, and N_HKUST604/14), Kaye Merlin Brutton Bequest from The University of Melbourne, the Innovation and Technology Commission (ITCPD/17-9) and the University Grants Committee of Hong Kong (AoE/P-03/08). N.Z., S.C., Y.H. and B.Z.T. thank the support from Natural Science Foundation of China (51403122), Endeavour Fellowship from Australia Government, McKenzie Fellowship from The University of Melbourne, and Guangdong Innovative Research Team Program (201101C0105067115), respectively.

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