

Specific Imaging and Tracking of Mitochondria in Live Cells by a Photostable AIE Luminogen

Chris W.T. Leung, Yuning Hong, and Ben Zhong Tang

Abstract

Tracking the dynamics of mitochondrial morphology has attracted much research interest because of its involvement in early stage apoptosis and degenerative conditions. To follow this process, highly specific and photostable fluorescent probes are in demand. Commercially available mitochondria trackers, however, suffer from poor photostability. To overcome this limitation, we have designed and synthesized a fluorescent agent, tetraphenylethene-triphenylphosphonium (TPE-TPP), for mitochondrial imaging. Inherent from the mitochondrial-targeting ability of TPP groups and the aggregation-induced emission (AIE) characteristics of the TPE core, TPE-TPP possesses high specificity to mitochondria, superior photostability, and appreciable tolerance to environmental change, allowing imaging and tracking of the mitochondrial morphological changes in a long period of time.

Key words Aggregation-induced emission, Photostable, Morphological change, Triphenylphosphonium

1 Introduction

Mitochondria, the organelle found in almost all eukaryotic cells, play a vital role in the life and death of cells. The most prominent function of mitochondria is to produce ATP, the energy currency of the cell. The production of ATP involves a series of electron transport systems in the oxidation phosphorylation pathway, which is also found to be associated with the generation of reactive oxygen species (ROS) [1, 2]. The production of ROS in mitochondria leads to propagation of free radicals, damaging of cells, and contribution to cell death, which is known as mitochondria-mediated apoptosis [3, 4]. The morphology of mitochondria, though varies upon cell type, cell-cycle stage, and intracellular metabolic state, is affected by and thus reflects cell functioning [5]. The morphology is controlled by a set of proteins, mutations of which will cause

several human diseases including degenerative diseases such as Parkinson's and Alzheimer's diseases [6]. Recent reports also show that proteins participating in apoptosis can affect the morphology of mitochondria [5, 7]. Tracking the mitochondrial morphological change may give insight for studying apoptosis and degenerative conditions.

Fluorescent probes that can selectively illuminate cellular mitochondria are powerful tools for monitoring the morphological changes and studying these processes. For observing the dynamic changes in certain period of time, the probe must be photostable under the continual irradiation of light from fluorescent microscopes. Conventional fluorescent dyes for mitochondria staining have been developed [8, 9]. Their photostability, however, leaves much to be desired. Very diluted solutions of these dyes are used in the imaging process and such small numbers of the dye molecules can be quickly photobleached when a harsh laser beam is used as the excitation light source. The photostability cannot be improved by using higher fluorophore concentration due to the accompanying concentration-quenching effect.

Triphenylphosphonium-functionalized aggregation-induced emission (AIE) luminogens are tackling those problems with high tolerance to changing microenvironment, photostability, and its AIE features. Their characteristics exhibit opposite phenomena: they are almost nonfluorescent when molecularly dissolved but become highly emissive in the aggregate state with fluorescence increasing along with the increase of fluorophore concentration [10]. Restriction of intramolecular motions (RIM) is proposed as the main cause of the AIE effect [11]. Lipophilic AIE molecules form nanoaggregates in aqueous solution spontaneously because of their hydrophobic nature [12]. These nanoaggregates have been successfully applied for long-term cell tracking in our previous work [13]. It is envisaged that the nanoaggregates of the AIE molecules would possess better photostability than single fluorescent molecule in dilute solutions.

In this chapter, AIE luminogens are decorated with mitochondria-targeting moieties to achieve higher specificity to mitochondria. Tetraphenylethene (TPE), an archetypal AIE luminogen, was synthesized and functionalized with triphenylphosphonium (TPP) groups (TPE-TPP, Fig. 1). TPP is a well-known functional group that facilitates the entrance of molecular probes into mitochondria by its lipophilicity and electrophoretic force [2]. We here demonstrate that TPE-TPP can light up mitochondria specifically in live cells (*see* Fig. 2) with superior photostability that enables the observation of mitochondrial morphological changes (*see* Fig. 3).

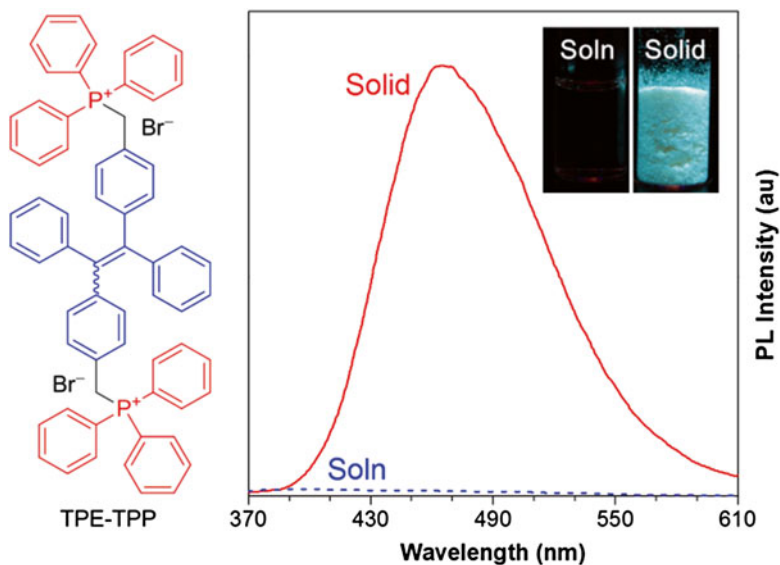


Fig. 1 Chemical structure of TPE-TPP; PL spectra of TPE-TPP in solid and solution (soln) states. *Inset:* Photographs of DMF solution (*left*) and solid powder (*right*) of TPE-TPP taken under UV irradiation. Concentration of TPE-TPP, 10 μ M; excitation wavelength, 321 nm

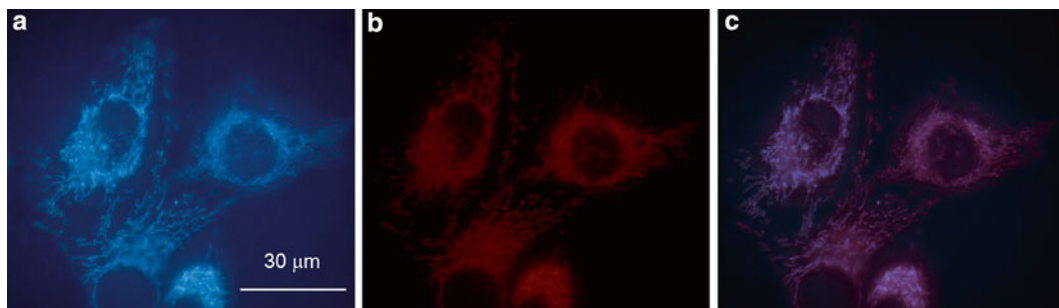


Fig. 2 Fluorescent images of HeLa cells stained with (a) TPE-TPP (5 μ M) for 1 h and (b) MitoTracker® Red FM (MT, 50 nM) for 15 min. (c) Merged images of panels (a) and (b). Excitation wavelength: 330–385 nm (for TPE-TPP) and 540–580 nm (for MT)

2 Materials

2.1 Equipment

1. NMR analysis was conducted on a Bruker ARX 400 spectrometer with tetramethylsilane (TMS; $\delta=0$) as internal standard.
2. Mass spectroscopy was carried out on a Finnigan TSQ 7000 triple quadrupole spectrometer operating in fast-atom bombardment (FAB) mode and API QSTAR XL System mode.
3. Fluorescence spectra were taken on a PerkinElmer LS 55 spectrofluorometer with a Xenon discharge lamp excitation.

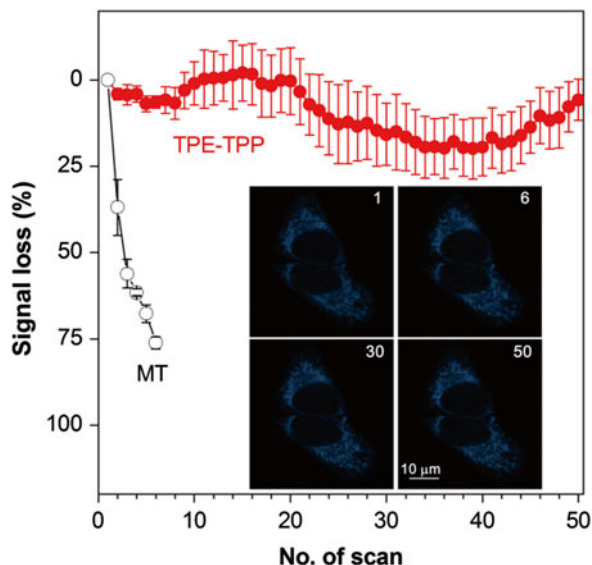


Fig. 3 Signal loss (%) of fluorescent emission of TPE-TPP (solid circle) and MT (open circle) with increasing number of scans. *Inset*: example of fluorescent images of living HeLa cells stained with TPE-TPP (5 μ M) with increasing number of scans (1–50 scans; the number of scan shown in the *upper right corner* of the panel). Excitation wavelength, 405 nm; emission filter, 449–520 nm; irradiation time, 7.75 s/scan

4. The HeLa cells were imaged under a fluorescence microscope (BX41 microscope; for TPE-TPP, λ_{ex} = 330–385 nm, dichroic mirror = 400 nm and emission filter = 420 nm long pass; for MT, λ_{ex} = 540–580 nm, dichroic mirror = 600 nm and emission filter = 610 nm long pass).
5. The photostability test was investigated by confocal microscope (Zeiss laser scanning confocal microscope; LSM7 DUO). TPE-TPP was excited at 405 nm (6 % laser power) and the fluorescence was collected at 449–520 nm. MT was excited at 560 nm (18 % laser power) and fluorescence was collected at 581–688 nm.

2.2 Reagents and Chemicals

THF (Labscan) was purified by simple distillation from sodium benzophenone ketyl under nitrogen immediately before use. Zinc dust, titanium(IV) chloride, *N*-bromosuccinimide (NBS), benzoyl peroxide (BPO), triphenylphosphine (PPh_3), benzaldehyde, 4-cyanobenzaldehyde, carbon tetrachloride, 4-nitrobenzaldehyde, triphenylamine, *N,N*-dimethylformamide (DMF), dichloromethane (DCM), dimethylsulfoxide (DMSO), and other reagents were all purchased from Aldrich and used as received.

2.3 Cell Culture

The following materials are used for culturing the HeLa cells.

1. Minimum essential medium, FBS, penicillin, and streptomycin were purchased from Gibco, Invitrogen.
2. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Sigma.
3. 35 mm culture dishes were purchased from Corning.
4. Phosphate-buffered saline (PBS).
5. Cover slides.
6. Cell culture CO₂ incubator.

3 Methods

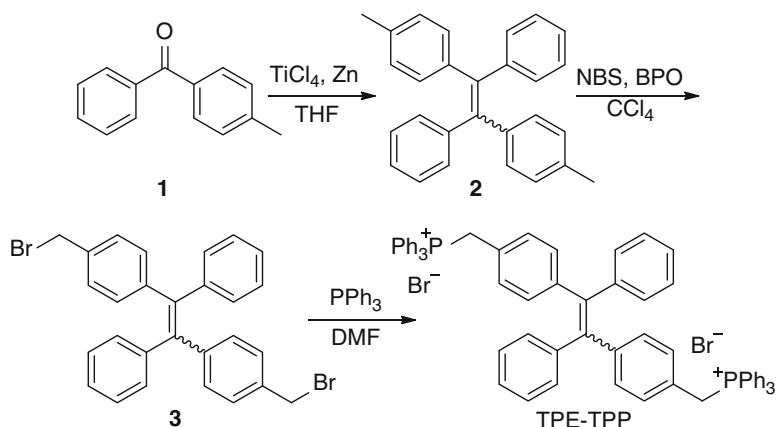
3.1 Synthesis (Scheme 1)

3.1.1 Synthesis of 1,2-bis(4-methylphenyl)-1,2-diphenylethane (2)

1. A suspension of 4-methylbenzophenone (1, 3.6 g, 10.0 mmol), TiCl₄ (1.9 g, 10.0 mmol), and Zn dust (1.3 g, 20.0 mmol) in dry THF (100 mL) was refluxed for 20 h.
2. Afterward, the reaction mixture was cooled to room temperature and filtered. The filtrate was evaporated and the crude product was purified on a silica-gel column using DCM as eluent.

3.1.2 Synthesis of 1,2-bis[4-(bromomethyl)phenyl]-1,2-diphenylethane (3)

1. To a mixture of 2 (1.8 g, 5.0 mmol) and NBS (1.7 g, 10.0 mmol) in CCl₄ was added catalytic amount of BPO at room temperature. The mixture was stirred and heated to reflux for 8 h.
2. After filtration and solvent evaporation, the product was purified by silica-gel chromatography using DCM/hexane (1:4 v/v) as eluent.



Scheme 1 Synthetic route to TPE-TPP

**3.1.3 Synthesis of bis
(triphenylphosphonium)
tetraphenylethene
(TPE-TPP)**

1. Triphenylphosphonium salt, TPE-TPP, was prepared from 3 (0.5 g, 1.0 mmol) and triphenylphosphine (1.0 g, 4.0 mmol) in DMF at 100 °C.
2. After stirring for 24 h, the solution was poured into large amount of toluene.

**3.2 Cell Imaging
by TPE-TPP**

3.2.1 Cell Culture

The HeLa cells were cultured in minimum essential medium containing 10 % fetal bovine serum and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin) in a 5 % CO₂ humidity incubator at 37 °C.

3.2.2 Cell Imaging

1. HeLa cells were grown overnight on a 35 mm petri dish with a coverslip.
2. The live cells were stained with 5 µM of TPE-TPP for 15 min, 30 min, or 2 h (by adding 2 µL of a 5 mM stock solution of TPE-TPP in DMSO to 2 mL culture medium) or 50 nM MitoTracker® Red FM (MT) for 15 min (by adding 0.5 µL of a 200 µM stock solution of MT in DMSO to 2 mL culture medium).
3. The live cells were washed with PBS three times after incubation with dyes.
4. Imaging medium (10 mM HEPES in MEM) was added to the dish.
5. The cells were observed under a fluorescent microscope through the observation window.

**3.2.3 Mitochondrial
Morphological Change
(Photostability Test)**

1. HeLa cells were grown overnight on a plasma-treated 25 mm round coverslip mounted to the bottom of a 35 mm petri dish with an observation window.
2. The live cells were stained with 5 µM of TPE-TPP for 30 min (by adding 2 µL of a 5 mM stock solution of TPE-TPP in DMSO to 2 mL culture medium) or 50 nM MitoTracker® Red FM (MT) for 15 min (by adding 0.5 µL of a 200 µM stock solution of MT in DMSO to 2 mL culture medium).
3. The live cells were washed with PBS three times after incubation with dyes.
4. 2 mL of imaging medium (10 mM HEPES in MEM) was added to the dish.
5. HeLa cells were then imaged by confocal microscope.
6. 10 µM CCCP (by adding 1 µL of a 200 mM stock solution of CCCP in DMSO to 2 mL culture medium) after the HeLa cells were imaged.
7. TPE-TPP-stained living HeLa cells were scanned for 50 times with 7.75 s/scan irradiation time.
8. MT-stained living HeLa cells were scanned for 7 times with 7.75 s/scan irradiation time.

4 Notes

1. Drill a hole of around 10 mm diameter in the middle of the dish. Place cover slide over the dish using paraffin.
2. When comparing the specificity of TPE-TPP and MT to mitochondria, MT should be excited before TPE-TPP as it is easily quenched by excitation wavelength.
3. Add the CCCP carefully into the dish after imaging by confocal microscope as the disturbance to the cells should be minimized for comparing the morphological change.
4. The power used for test photostability of TPE-TPP and MT should be unified for fair measurement.

Acknowledgments

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