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Novel fluorescent probes for highly selective two-photon imaging of mitochondria in living cells

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ABSTRACT

The synthesis and characterization of a pair of novel pyridine cation derivatives possessing two-photon excitation fluorescence (TPEF) properties and selectively staining mitochondria in living SiHa cells within 30 min, CAI and CAEI, were reported. And the green-emitting CAI displayed a much larger two-photon excitation fluorescence action absorption cross-section ($\delta \times \Phi$) of 328 g at 860 nm in comparison with commercial MitoTracker Green (MTG) with maximum $\delta \times \Phi$ value of 2.18 g at 850 nm. As is known to all, $\delta \times \Phi$ is a crucial parameter to obtain a high-quality microscopic photo in living cells in two-photon microscopy (TPM). Moreover, the fact that the co-localization coefficient between CAI and conventional MitoTracker Red (MTR) was 0.95 in SiHa cells demonstrated specific staining performance of CAI to mitochondria. As biosensors, both CAI and CAEI possessed a number of beneficial properties such as large $\delta \times \Phi$ and Stokes shifts, good membrane permeability, long retention time, high photostability and excellent counterstain compatibility with different biosensors for instance Hoechst 33342 and *D* 307, which ranked them as one of the best TPEF mitochondrial probes. Furthermore, related mechanism research suggested that their localization properties were dependent on the mitochondrial membrane potential in living cells. And their remarkable properties can extend the investigation on mitochondria in a biological context.

1. Introduction

Mitochondria are membrane-bound organelles with 0.5 to 10 μm diameter that form the cellular power plant (Sanadi, 1965). At the same time, they play a fundamental role in numerous physiological processes in cells, such as cell differentiation (Folmes et al., 2012), apoptosis (Patrice et al., 1996), signal transduction (Rizzuto et al., 2012) and reactive oxygen species generation (Murphy, 2009). The most vital function of mitochondria is for respiration and to provide energy for cellular survival and proliferation (Sanadi, 1965). Hence, the majority of eukaryotic cells possess abundant mitochondria, and their size, number and morphology vary with the metabolic state of the cell, the cell cycle, and cellular development, differentiation and pathological states (Rouiller, 1960). In particular, researchers have suggested that mitochondrial morphology and number have close connection with many diseases, such as cancer (Gogvadze et al., 2008; Kroemer, 2006), Alzheimer's disease (Du et al., 2010). Thus, investigations to the mitochondrial structure and function are of ultimate importance for our understanding of related physiological processes as well as diagnostics and therapeutics of corresponding diseases.

Fluorescence imaging techniques combined with the probes with selectivity for an entity provide powerful tools for biological research and have been utilized in various studies for mitochondria (Neto et al., 2013). In particular, TPM with revolutionary development in fluorescence imaging techniques, based on the unique characteristics of the two-photon absorption process that occurs in the probes excited, such as long excitation wavelength (700 to 1000 nm) and quadratic-intensity dependence (Göppert, 1931; Kaiser and Garrett, 1961), enables the high-resolution visualization of intracellular organelles in thick, opaque, living specimens (Gerritsen and De Grauw, 1999; Potter, 1996; Reynolds and Drexhage, 1975). However, the advantages of TPM, including high spatial resolution, deep penetration, low photodamage, no image distortion and reduced photobleaching (Chinta et al., 2012; Cho et al., 2011; Tao et al., 2012), can be severely compromised if the $\delta \times \Phi$ of the extrinsic fluorophores used as biosensors are smaller than or similar to the endogenous fluorophores. Theoretically, as one-photon excitation and two-photon excitation obey different selection rules (Göppert, 1931; Kaiser and Garrett, 1961),

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one cannot expect conventional one-photon probes to necessarily have optimized properties for two-photon excitation. On the other hand, two-photon absorption materials with large $\delta \times \Phi$ values usually possess extense π-conjugated planar structures, which are often not compatible with biological specimens. Thus, the design and synthesis of novel fluorescent probes with large $\delta \times \Phi$ values and good biocompatibility is a fundamental and vital work for researchers in biosensor.

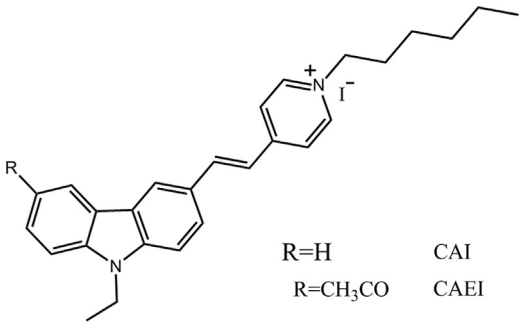
As a biosensor, its selectivity to the target is one of vital parameters, namely, the probe must interact selectively with an entity such as an ion, a molecule, organelle or other structure. For a new probe, its co-localization coefficient with a conventional counterpart in a standard double-staining experiment can demonstrate primely the selectivity of a newcomer (Miao et al., 2013). MTR or MTG should be right specimens to detect the selectivity of a novel mitochondrial fluorescent probe.

To the best of our knowledge, although a wide variety of mitochondrial fluorescent probes are available commercially, only Rhodamine has been applied in TPM (Monteith et al., 2013). So far, high selective TPEF probes for mitochondria (Han et al., 2012; Meulenaere et al., 2012; Yang et al., 2013) are still rare. More interestingly, the majority of these reported probes use a triphenylphosphine moiety as a targeting group for mitochondria, but other chemical structures with high selectivity to mitochondria have seldom been reported. Therefore, a novel TPEF probe possessing a large $\delta \times \Phi$ value, high selectivity to mitochondria, good biocompatibility, and a novel targeting group for mitochondria is extremely desirable.

Previously, our group has reported a series of TPEF mitochondrial probes based on the donor-π-acceptor (D-π-A) structure, and they exhibited a large $\delta \times \Phi$ value and low toxicity (Liu et al., 2011). Unfortunately, the membrane permeability of these probes was not confirmed, since they could image living cells only after overnight incubation.

More recently, Yang et al. (2013) developed a new TPEF probe with a triphenylphosphine moiety for mitochondrial fluorescence imaging. And their co-stained experiment results with Mito tracker indicated that the probe reported can exclusively label mitochondria. But the co-localization coefficients of the probe with conventional mitochondrial probes were not studied. At the same time, the incubation time (24 h) of this probe for the internalization of the dyes was long compared to that of other cell-permeant dyes which require less than 30 min in general.

In this ongoing study, we designed and synthesized two novel pyridine cation derivatives with a hexyl chain, CAI and CAEI (Scheme 1), as mitochondrial TPEF probes. Both can penetrate the cell membrane and enter a living cell within only half an hour. At the same time, that the co-localization coefficients with MTR were 0.95 for CAI and 0.88 for CAEI demonstrated that this pair of molecules possess high selectivity for intracellular mitochondria. In comparison with two commercial mitochondrial probes, MTR and MTG, CAI and CAEI also displayed a much larger $\delta \times \Phi$ value and emitted detectable TPEF signals when imaging living cells in TPM. Furthermore, various merits of CAI and CAEI, such as large Stokes shifts, long retention time, high photostability and excellent counterstain compatibility with Hoechst 33342 and D 307 can rank them among the top mitochondrial TPEF probes. In addition, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) experiment results suggested that their staining ability to mitochondria is dependent on the mitochondrial membrane potential in living cells.



Scheme 1. Structures of CAI and CAEI.

2. Experimental

2.1. Apparatus and general methods

The UV–visible–near-IR absorption spectra of dilute solutions were recorded on a HITACH U-2910 spectrophotometer. One-photon fluorescence spectra were obtained on a HITACH F-2700 spectrofluorimeter equipped with a 450-W Xe lamp. TPEF was measured on a SpectroPro300i and the pump laser beam came from a mode-locked Ti:sapphire laser system at the pulse duration of 200 fs, a repetition rate of 76 MHz (Coherent Mira900-D). PBS buffer solution:10 mM NaCl, $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, pH = 7.40.

2.2. Synthesis and characterization

CAI was prepared in four facile steps according to the synthesis route in Supplementary material. Reaction of carbazole and bromoethane afforded 9-ethyl-carbazole. A solution of 9-ethyl-carbazole and *N*-bromosuccinimide (NBS) in CH_3Cl was added in acetic acid and stirred for 24 h to afford 3-bromo-9-ethyl-carbazole. Under argon 3-(4-vinylpyridine)-9-ethyl-carbazole was obtained through a heck reaction using the reagents 3-bromo-9-ethyl-carbazole and 4-vinylpyridine. After refluxing 3-(4-vinylpyridine)-9-ethyl-carbazole with excess iodohexane in acetone for overnight, the crude product was collected as powder. After recrystalization from methanol, CAI was obtained. Synthesis of CAEI was analogous: 9-ethyl-carbazole proceeded to an F–C (Friedel–Crafts) reaction with acetic anhydride in anhydrous methylene dichloride to afford 3-acetyl-9-ethyl-carbazole. Next, analogous procedures with preparation of CAI have been adopted to synthesize CAEI. Experimental details of the synthesis and characterization of the compound can be found in Supplementary material.

2.3. Measure of fluorescence quantum yield and two-photon absorption cross sections

The luminescence quantum yields can be calculated by means of Eq. (1):

$$\Phi_s = \Phi_r \left(\frac{A_r(\lambda_r)}{A_s(\lambda_s)}\right)\left(\frac{n_s^2}{n_r^2}\right)\frac{F_s}{F_r} \quad (1)$$

where the subscripts *s* and *r* refer to the sample and the reference materials, respectively. Φ is the quantum yield, *F* is the integrated emission intensity, *A* stands for the absorbance, and *n* is the refractive index. In this work, the quantum yields were calculated by using fluorescein in sodium hydroxide aqueous solution (pH = 13, Φ = 95%) (Demas and Crosby, 1971) as a standard.

Two-photon absorption cross sections have been measured using the two-photon induced fluorescence method, and thus the cross section can be calculated by means of Eq. (2) (Xu and Webb, 1996):

$$\delta_s = \delta_r \frac{\Phi_r c_r n_r F_s}{\Phi_s c_s n_s F_r} \quad (2)$$