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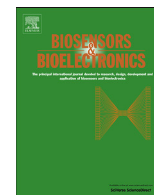

Ultrasensitive fluorescent ratio imaging probe for the detection of glutathione ultratrace change in mitochondria of cancer cells

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ABSTRACT

Glutathione (**GSH**) ultratrace change in mitochondria of cancer cells can mildly and effectively induce cancer cells apoptosis in early stage. Thus, if **GSH** ultratrace change in mitochondria of cancer cells could be recognized and imaged, it will be beneficial for fundamental research of cancer therapy. There have reported a lot of fluorescent probes for **GSH**, but the fluorescent probe with ultrasensitivity and high selectivity for the ratio imaging of **GSH** ultratrace changes in mitochondria of cancer cells is scarce. Herein, based on different reaction mechanism of sulfonamide under different pH, a sulfonamide-based reactive ratiometric fluorescent probe (**IQDC-M**) was reported for the recognizing and imaging of **GSH** ultratrace change in mitochondria of cancer cells. The detection limit of **IQDC-M** for **GSH** ultratrace change is low to 2.02 nM, which is far less than 1.0‰ of endogenic **GSH** in living cells. And during the recognition process, **IQDC-M** can emit different fluorescent signals at 520 nm and 592 nm, which results in it recognizing **GSH** ultratrace change on ratio mode. More importantly, **IQDC-M** recognizing **GSH** ultratrace change specifically occurs in mitochondria of cancer cells because of appropriate water/oil amphipathy (log P) of **IQDC-M**. So, these make **IQDC-M** possible to image and monitor **GSH** ultratrace change in mitochondria during cancer cells apoptosis for the first time.

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1. Introduction

Glutathione (**GSH**, γ-L-glutamyl-L-cysteinyl-glycine) is one of the most important tripeptides in living cells. It is known as a substrate in both conjugation reactions and reduction reactions, catalyzed by glutathione *S*-transferase enzymes in mitochondria (Ballatori et al., 2009; Cavalca et al., 2009). **GSH** in mitochondria, especially in mitochondria of cancer cells, play many key roles in the biological process. For example, **GSH** ultratrace changes could induce cancer cells apoptosis in early stage (Mohamed et al., 1998; Xu and Thornalley, 2001). Thus, to design and synthesize a smart fluorescent probe or sensor for the detecting and imaging of **GSH** ultratrace changes in mitochondria of cancer cells, whilst sparing normal cells, would provide an effective visual tool for the fundamental research of cancer cells apoptosis in early stage (Wen et al., 2002).

In the past years, many fluorescent probes or sensors for **GSH** were designed based on energy transfer systems. They could emit different fluorescence signals, for instance, ratio fluorescence signal, off-on fluorescence signal and so on, when they encountered with **GSH** (Ahn et al., 2007; Chang et al., 2016; Chen et al., 2015; Fujikawa et al., 2008; Guo et al., 2012; Lim et al., 2014; Liu et al., 2014a, 2014b; Niu et al., 2015; Sun et al., 2014; Wang et al., 2014, 2016; Xu et al., 2013, 2015; Yu et al., 2013; Yang et al., 2013, 2014; Yin et al., 2014, 2015; Zhang et al., 2016). Because of the high resolution and real-time monitor capabilities of such probes or sensors, they have been used to image **GSH** in vivo and in vitro. But, some probes or sensors mentioned above presented non-selectivity for **GSH**, if other compounds containing the thiol exist in vivo and in vitro, such as homocysteine (**Hcy**) and cysteine (**Cys**). Furthermore, some probes or sensors no possessed the ultrasensitivity for **GSH** ultratrace change. These have limited applications of these probes or sensors in imaging of **GSH** ultratrace changes. More regrettably, these fluorescent probes or sensors have a common limitation. That is, none of them could specifically recognize **GSH** ultratrace changes in mitochondria of cancer cells.

With these in mind, herein, we reported a ratiometric fluorescent probe (**IQDC-M**) with high selectivity and ultrasensitivity

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for the recognizing and imaging of **GSH** ultratrace change in mitochondria of cancer cells. Based on different reaction mechanism between sulfonamide and different level of **GSH** changes (Mohamed et al., 1998; Xu and Thornalley, 2001), **IQDC-M** has been designed to become a sulfonamide derivative. To improve high selectivity of **IQDC-M** for **GSH** ultratrace changes, "S-N" bond length in sulfonamide group was further modulated (Yin et al., 2014). Furthermore, to realize the recognition process of **GSH** changes only occurring in mitochondria of cancer cells, the water/oil amphipathy (log P) of **IQDC-M** was adjusted by introducing a phosphor salt (Xu et al., 1996). To image **GSH** changes on ratio fluorescence mode, two fluorophore (**IQ-M** and **DC**) as energy donor and acceptor were introduced into **IQDC-M**, respectively. And **IQ-M** and **DC** were linked by sulfonamide group to realize fluorescence resonance energy transfer (FRET). Upon this design strategy, we envision that **IQDC-M** would recognize and image **GSH** ultratrace changes in mitochondria of cancer cells. And I expect that it could be as a visual tool for real-time monitoring and imaging of **GSH** ultratrace changes in mitochondria during cancer cells apoptosis process.

2. Material and methods

2.1. Ratiometric fluorescent probe preparation

IQDC-M and intermediate products were synthesized on the basis of the route shown in Scheme S1. Thereinto, intermediate products, **IQ-M** and **DC** were synthesized from naphthalene derivatives in 70% and 89% yield (Scheme S1). **IQDC-M** was obtained by the sulfonylation reaction between **IQ-M**, **DC** and thionyl chloride (Yin et al., 2014). **IQDC-M** and intermediate products were clearly characterized by NMR (Avance 400 MHz spectrometer, Bruker Co., Switzerland) and HRMS (Bruker microToF II, Bruker Co., Switzerland; Attached Fig. in Supporting Information). Unless otherwise noted, the AR grade of solvents and chemicals were used in this work. To purify compounds by column chromatography, silica gel (100–200 mesh) was used as the stationary phase. **GSH** was purchased from Sigma Chemical Co. (USA). In all spectroscopic experiments, water was purified by using a Milli-Q system.

2.2. Quantum calculations

Quantum chemical calculation carry out by Gaussian 09. The density functional theory (DFT) with RB3LYP/6-31G* level were used to calculate electronic transition energies and corresponding oscillator strengths.

2.3. Cell culture and staining with probes

NIH3T3, HepG 2, MCF-7, CHO, and HeLa cell lines were obtained from Institute of Basic Medical Sciences (IBMS) of the Chinese Academy of Medical Sciences. Dulbecco's Modified Eagle's Medium (DMEM, WelGene) and eagle's minimum essential medium (MEM, WelGene) supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS; Gibco) were used for culture cells in a 5.0 wt% /vol CO_2 incubator at 37 °C. Cell lines were seeded into a 35 mm dish with 20 mm well glass bottomed dish (MatTek) one day before imaging. And than, the cells were stained with **IQDC-M.**

2.4. Confocal microscopic imaging

Olympus spectral confocal multiphoton microscope (FV1200) with mode-locked titanium-sapphire laser source (MaiTai, Spectra-Physics, USA) was used to image cell on one/two-photon mode. These imaging results were obtained at the following parameters: internal PMTs are at 16 bit and 1024 × 1024 pixels, excitation wavelength: 488 nm or 800 nm; scan range: 520 ± 20 nm (green channel), 592 ± 20 nm (red channel).

2.5. Flow cytometry and FITC-Annexin V/PI method

NIH3T3, MCF-7, CHO, and HeLa cell lines were incubation with probes (0.50 μM) for 30 min, and then, they were dispersed into PBS solution at level of 10,000 cells/500 μL. Samples were analyzed by flow cytometer (BD FACSCanto II, USA). The average fluorescence intensity in 10,000 cells was obtained and analyzed with BD FACSDiva software.

Wash cells twice with cold BioLegend's Cell Staining Buffer, and then resuspend cells in Annexin V Binding Buffer at a concentration of 0.25–1.0 × 10^7 cells/mL. Transfer 100 μL of cell suspension in a 5.0 mL test tube. Add 5.0 μL of FITC Annexin V. Add 10 μL of Propidium Iodide Solution. Gently vortex the cells and incubate for 15 min at room temperature (25 °C) in the dark. Add 400 μL of Annexin V Binding Buffer to each tube. Analyze by flow cytometry with proper machine settings.

2.6. Mitochondrial membrane potential assay

JC-1 Mitochondrial Membrane Potential Assay Kit can be used to study the behavior of mitochondria in a variety of conditions, including apoptosis. The main advantage of this assay is that the changes in $\Delta\Psi_m$ reflected by different forms of JC-1 as either green or red fluorescence can be both qualified and quantified by fluorescence microscopy, flow cytometry, or a fluorescence plate reader with appropriate filter sets. Thaw the JC-1 Reagent at room temperature. Mix well. To avoid repeated freeze/thawing of this solution, we recommend that you make small aliquots and store them at −20 °C.

Assay Buffer Preparation: Dissolve three Cell-Based Assay Buffer tablets in 300 mL of distilled water. This buffer should be stable for approximately one year at room temperature.

JC-1 Staining Solution Preparation: Thaw an aliquot of the JC-1 Reagent at room temperature. Prepare a staining solution by diluting the reagent 1:10 in the culture medium you are using for your cells. Mix well to make sure there are no particles or flakes in the solution. Culture cells in 6-, 12-, or 24-well plates at a density of 5.0 × 10^5 cells/mL in a CO_2 incubator overnight at 37 °C. Treat the cells with or without experimental compounds (each sample should be run in duplicate or triplicate). Incubate the cells according to your normal protocol. Add 100 μL of the JC-1 Staining Solution per mL of culture medium to each well of the plate. For example, if you culture cells in 2.0 mL of culture medium in a 6-well plate, add 200 μL of the JC-1 Staining Solution into each well. Mix gently. Further dilution, such as adding only 50 μL of JC-1 Staining Solution to 1.0 mL of culture medium, may be used in cases when the staining is too intense.

Incubate samples in a CO_2 incubator at 37 °C for 15–30 min. Sufficient staining is usually obtained after 15 min of incubation. Harvest cells from each well into a plastic tube fitted for the flow cytometer. The samples can be directly analyzed in the culture medium.

Analyze the samples immediately. Healthy cells with functional mitochondria contain red JC-1 KA1324 6/10 J-aggregates and are detectable in the FL2 channel. Apoptotic or unhealthy cells with collapsed mitochondria contain mainly green JC-1 monomers and are detectable in the FL1 channel.

2.7. Glutathione assay

Set a plate reader to 412 nm with kinetic read at 1 min intervals

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