Biosensors and Bioelectronics 78 (2016) 344–350

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

# Thrombin-mediated ratiometric two-photon fluorescent probe for selective imaging of endogenous ultratrace glutathione in platelet

Hua Zhang [a,b,*], Caixia Wang [a,b], Ge Wang [c], Kui Wang [a,b], Kai Jiang [a,b,*]

[a] Collaborative Innovation Center of Henan Province for Green Manufacturing of Fine Chemicals, Key Laboratory of Green Chemical Media and Reactions, Ministry of Education, Henan Normal University, Xinxiang, Henan 453007, PR China

[b] Henan Key Laboratory of Green Chemical Media and Reactions, School of Chemistry and Chemical Engineering, Henan Normal University, Xinxiang, Henan 453007, PR China

[c] Xinxiang Medical University, 601 Jinsui Road, Hongqi Zone, Xinxiang 453000, PR China

ARTICLE INFO

*Article history:*
Received 6 October 2015
Received in revised form
12 November 2015
Accepted 23 November 2015
Available online 30 November 2015

*Keywords:*
Two-photon fluorescent probe
Ultratrace glutathione
Platelet
Endogenous
Ratiometric imaging

ABSTRACT

Ultratrace change of reduced glutathione (**GSH**) can weaken coagulation function of platelet (**PLT**). Thus, rapid and sensitive imaging of **GSH** specific in **PLT** is beneficial for monitoring coagulation function of **PLT**. Many fluorescent probes for **GSH** have been reported, but ratio fluorescent probe with excellent two-photon property for screening **PLT** from peripheral blood and quantitative imaging of **GSH** are scarce. In this work, a thrombin-mediated two-photon **GSH**-specific fluorescent probe (**IQDC-L**) was reported. Sulfuric diamide, a key group as linker, was introduced into **IQDC-L**, which resulted in not only specific selectivity for **GSH**, but also FRET occurring in probe. When **IQDC-L** encountered **GSH**, "S–N" in sulfonamide group was cut off, and FRET was inhibited. Furthermore, fluorescence intensities at 520 and 595 nm presented linear change on ratio mode in the range of **GSH** (2.0–65 nM). The lowest detection for **GSH** was as low as 0.083 nM. Intriguingly, **IQDC-L** under thrombin-mediated was able to screen **PLT** from peripheral blood without any interference. Thus, **IQDC-L** could be used to screen **PLT** from peripheral blood, and simultaneously, to in situ image ultratrace **GSH**.



## 1. Introduction

Glutathione (**GSH**) is one of the widely existing antioxidant substances in bio-systems (Economou et al., 2003; Passweg and Tichelli, 2009), and its concentration in human peripheral blood, individual cells and organelles vary from millimole-level to micromole-level, even to nanomole-level. For example, the contents of **GSH** in human peripheral blood (Rahman et al., 2006), epithelia lining fluid (Rahman et al., 2006), **PLT** (Thomas and Skrinska, 1985) and human umbilical vein endothelial cells (Kokura et al., 1999) are roughly 2–3 mM, 400–600 μM, 30 nM, and 4 nM, respectively. Owing to its reducibility, **GSH** was usually used to weaken oxidative stress in bio-systems, including in **PLT** (Meister and Anderson, 1983; Niu et al., 2012; Shao et al., 2010; Yin et al., 2014). Recently, clinical data indicated that **GSH** can eliminate oxide which comes from self-metabolism process in **PLT**, to maintain the activity of **PLT** in regular range (Thomas and Skrinska, 1985). And only ultratrace changes of **GSH** in **PLT** may increase the risks of atherosclerosis, even lead to suffering from thrombus, because that its content in **PLT** is already at nanomole-level (Anthea et al., 1993; Maton et al., 1997; Niu et al., 2012; Shao et al., 2010; Stewart and Wild, 2014; Vardiman et al., 2009; Yin et al., 2014). Thus, if very low level of **PLT** ($100 \times 10^9\ L^{-1}$) could be non-destructively screened from peripheral blood and simultaneously imaged ultratrace changes **GSH** in them, then the related diseases could be early warning, and treatment plan may be provided in early stage (Meister and Anderson, 1983).

In recent years, fluorescent probes or sensors present exciting opportunities for detecting and imaging of specific substances in bio-systems, such as **GSH**. But, other thiol compounds existing in bio-systems, such as, cysteine (**Cys**) and homocysteine (**Hcy**), would frequently interfere with detection of **GSH**. According to the different between **GSH**, **Cys** and **Hcy**, many research groups (Ahn et al., 2007; Chen et al., 2015; Fujikawa et al., 2008; Guo et al., 2012; Lim et al., 2014; Liu et al., 2014a, 2014b; Sun et al., 2014; Wang et al., 2014; Xu et al., 2013, 2015; Yu et al., 2013; Yang et al., 2013, 2014; Zhang et al., 2011) designed a series of fluorescent probes for specific detecting and imaging of **GSH**. These probes showed extraordinary optical and biological activities, such as near-infrared fluorescence, ratiometric fluorescence. However,

* Corresponding authors at: Collaborative Innovation Center of Henan Province for Green Manufacturing of Fine Chemicals, Key Laboratory of Green Chemical Media and Reactions, Ministry of Education, Henan Key Laboratory of Green Chemical Media and Reactions, School of Chemistry and Chemical Engineering, Henan Normal University, Xinxiang, Henan 453007, PR China.

*E-mail addresses:* zhanghua1106@163.com (H. Zhang), jiangkai6898@163.com (K. Jiang).

http://dx.doi.org/10.1016/j.bios.2015.11.065
0956-5663/

none of these probes without interference could screen **PLT** from peripheral blood and simultaneously image nanomole-level of **GSH** in them on ratio mode. Thus, these have limited applications of probes in disease early warning.

Herein, based on fluorescence resonance energy transfer (FRET), we report a smart ratio two-photon fluorescent probe, **IQDC-L**, which could efficiently screen **PLT** from peripheral blood and as well as image nanomole-level of **GSH** in them. In molecular designing, sulfonamide groups were introduced into molecule, resulting in not only the specific selectivity for **GSH** over **Cys** and **Hcy** but also FRET occurring in donor (**IQ-L**) and acceptor (**DC**). Furthermore, fluorescence of **IQDC-L** had apparently different intensities at two sites with different amount of **GSH**. In the absence of **GSH**, **IQDC-L** mainly emitted fluorescence at 595 nm. However, dramatic fluorescent changes occurred when **IQDC-L** reacted with **GSH**, that is, the probe exhibited new green fluorescence at 520 nm, and they intensity gradually increased with concentration of **GSH** increasing. Results from in silico structural analyses and the outcome of whole-cell assays demonstrated that **IQDC-L** could specifically image ultratrace change of **GSH** on ratio mode. More importantly, **IQDC-L**, which could combine thrombin (Bode, 2006) only expressing in **PLT**, successfully screen **PLT** from peripheral blood. To the best of our knowledge, **IQDC-L** is the first two-photon fluorescent probe without interference for screening **PLT** from peripheral blood and simultaneous imaging of **GSH** ultratrace change in them on ratio mode.

## 2. Material and methods

### 2.1. GSH-specific probe preparation

Synthetic routes of **IQDC-L** and related intermediates and their characterization are listed in SI. The solvents and reagents (AR grade) were used in this work. Column chromatographic was used to purify compounds and silica gel (100–200 mesh) was used as fillers. **GSH** was obtained from Sigma Chemical Co. (USA). Doubly purified water was used in all experiments, which was prepared using by a Milli-Q system. 1.0 mM DMSO solution of **IQDC-L** as stock was used in spectrographic determination and cell experiments.

ESI-MS (Bruker microToF II, Bruker Co., Switzerland) with an auto sampler operated in-line with a quantum triple quadruple instrument was carried out in mass spectral studies on ESI positive or negative ion mode. NMR spectra were obtained from Avance 400 MHz spectrometer (Bruker Co., Switzerland).

### 2.2. Quantum calculations

Gaussian 09 was used in quantum chemical (Frisch et al., 2009), and the work of Han was as reference for setting up calculations parameter (Zhou et al., 2007). The density functional theory (DFT) (Dreizler et al., 1990) with B3-LYP and B3LYP-D3 functional were used in the geometry optimizations of the dyes. And 6–31G* basis set was utilized. No constraints to bonds/angles/dihedral angles were applied in the calculations and all atoms were free to optimize. The time-dependent density functional theory (TD-DFT) (Gross and Kohn, 1985; Stratmann et al., 1998) at the B3LYP/6-31G** level was used to calculate electronic transition energies and corresponding oscillator strengths.

### 2.3. Blood cell preparation and staining with probes

Blood components containing red blood cells, white blood cells and **PLTs**, respectively, were obtained from mice (Kunming, male, rat age: five weeks). And then, they were dispersed in PBS solution, and were incubated with probes at 37 °C in 5 wt%/vol $CO_2$ for 0.5 h. After 0.5 h, every blood component was prepared into relevant smear, respectively.

### 2.4. One/two-photon fluorescence 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 1600 × 1600 pixels, and scan speed is 400 Hz. The images and data were obtained from replicate experiments ($n=5$).

### 2.5. Measurement of two-photon cross section

The femtosecond (fs) fluorescence measurement technique was used to detect the two-photon absorption cross section ($\delta$) of the probe. The probe was dissolved in different solvents at a concentration of 0.5 mM, and then the two-photon induced fluorescence intensity was obtained under excitation at 690–920 nm. Fluorescein (0.8 μM, pH 11.0) was used as the reference. The intensities of the two-photon induced fluorescence spectra of the reference and sample resulting from the same excitation wavelength were determined, respectively. $\delta$ was calculated by using eq:

$$\delta = \delta_r(S_s\Phi_r\varphi_r c_s)/(S_s\Phi_r\varphi_r c_s)$$

In this equation, the sample and reference molecules were expressed as the subscripts s and r. $S$ denoted the intensity of the signal which was obtained by a CCD detector. Fluorescence quantum yield were expressed as $\Phi$, and the overall fluorescence collection efficiency of the experimental apparatus were expressed as $\varphi$. In addition, $c$ represented the concentration of the sample and reference molecules in solution, respectively. The measurement temperature was 25 ± 0.5 °C. The data were obtained from replicate experiments ($n=5$). In the figure, $\Phi\delta$ as $Y$-axis and corresponding excitation wavelengths as $X$-axis were used, respectively.

### 2.6. Flow cytometry

Blood cells get from mice (Kunming, male, rat age: two and five weeks). After incubation with probes (0.5 μM) and Hochest 33258 (1.0 μM) for 30 min, then blood cells were washed. And they were dispersed into PBS solution at level of 10,000 cells/500 μL. Samples were analyzed with the laser (405/488 nm) on a flow cytometer (BD FACSCanto II, USA). The average fluorescence intensity in 10,000 cells was obtained and analyzed with BD FACSDiva software. **IQDC-L**: excitation wavelength = 488 nm, scan range = 520 ± 20 nm, 595 ± 20 nm; Hochest 33258: excitation wavelength = 405 nm, scan range = 400–440 nm).

## 3. Results and discussion

### 3.1. Design and synthesis strategy of fluorescent probe

This work focused on designing a two-photon fluorescent probe for screening **PLT** from peripheral blood without interference and in situ imaging of ultratrace **GSH** in them on ratio mode. To screen **PLT** from peripheral blood, probe must be designed based on the most remarkable differences between **PLT** and peripheral blood, that is, trilamellar membrane and thrombin (EC: 3.4.21.5). Consider these differences, 1-methyl piperazine was