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A ratiometric two-photon fluorescent probe for cysteine and homocysteine in living cells

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ABSTRACT

A ratiometric two-photon fluorescent probe (**MQ**) was designed and synthesized to detect cysteine (Cys) and homocysteine (Hcy) with high selectivity. **MQ** exhibited a large two-photon absorption cross-section at 710 nm (265 GM). Significant blue-shifts of both fluorescent and UV–vis spectra of **MQ** were found upon the addition of Cys or Hcy. **MQ** responded to Cys/Hcy much faster than previous reported two-photon fluorescent probes for Cys/Hcy. The response mechanism was proved by ^{1}H NMR, Maldi-TOF MS and theoretical calculation. Cell cytotoxicity and bio-imaging studies revealed that **MQ** was cell-permeable and could be used to detect Cys with low cytotoxicity under two-photon excitation.

1. Introduction

Cysteine (Cys) and homocysteine (Hcy) are essential in biological systems [1]. The abnormal levels of these biothiols have been linked to a number of diseases, such as slowed growth, hair depigmentation, edema, lethargy, liver damage, muscle and fat loss, skin lesions, weakness and Alzheimer's disease [2]. Therefore, it is of high interest to develop detection and quantification methods for Cys/Hcy *in vitro* and *in vivo*. Among which, fluorescent probes with high sensitivity and selectivity for Cys/Hcy in living cells and tissues have attracted growing attentions over past years [3].

Numbers of fluorescent probes for Cys/Hcy have been developed based on specific reactions between probes and biological thiols such as Michael addition [4], cleavage reactions [5], and cyclization reaction [6]. Recently, two-photon microscopy (TPM) has been widely used in biomedical researches for its remarkable advantages over one-photon microscopy (OPM), including increased penetration depth, localized excitation and prolonged observation time [7]. In responding to the TPM technique, many two-photon fluorescent probes for detecting biological agents have been developed [8]. However, two-photon probes for Cys/Hcy were rarely reported. Among the reported two-photon probes for Cys/Hcy [9], drawbacks such as delayed response, poor cell-permeability and small two-photon absorption cross-section limited their application in living cells and tissues.

Herein, we report a ratiometric fluorescent probe (**MQ**, Scheme 1) for detecting Cys/Hcy based on 6-substituted quinoline, an excellent two photon-fluorescent group for cell imaging that was previously developed by our group [10]. The aldehyde group, as the specific reaction point for Cys/Hcy, was linked directly to the substituted quinoline skeleton. **MQ** showed excellent two-photon fluorescence as a result of intramolecular charge transfer (ICT) [11]. We believe that the aldehyde group in **MQ** can form thiazolidine and thiazinane with Cys/Hcy through cyclization reaction. The ICT process in the system will then be switched off and detection signals (*i.e.* the shift of the fluorescent spectrum along with the color change) will be obtained (Scheme 1).

2. Experimental

2.1. General procedures

All reagents and solvents were commercially purchased. All reactions were magnetically stirred and monitored by thin layer chromatography (TLC). Flash chromatography (FC) was performed using silica gel 60 (200–300 mesh). ^{1}H NMR spectra were recorded on Bruker-400 MHz spectrometers and ^{13}C NMR spectra were recorded on 100 MHz spectrometers. Fluorescence spectra were

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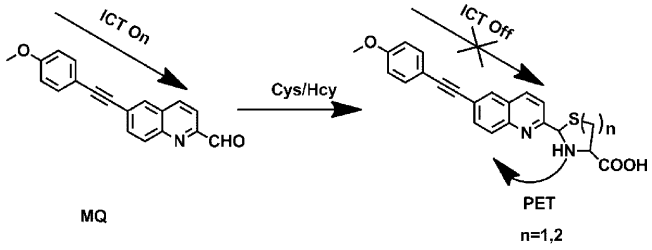


Scheme 1. Proposed response mechanism of **MQ** to Cys/Hcy.

obtained using a HITACHIF-2500 spectrometer. UV–vis absorption spectra were recorded on a Tech-comp UV 1000 spectrophotometer. MS spectra were conducted by Bruker autoflex III MALDI TOF mass spectrometer. The test solution of **MQ** (25 μM) in pH 7.4 PBS (containing DMSO/PBS, 9:1, v/v) was prepared. The solutions of various testing species were prepared from Hcy, Cys, Phe, Gly, Leu, Ala, Ser, Arg, Gly, GSH, Met, Gln, Lys and Glucose. The resulting solution was shaken well and incubated for 30 min at room temperature before recording the spectra.

2.2. Measurement of two-photon absorption cross-section (δ)

Two-photon excitation fluorescence (TPEF) spectra were measured using femtosecond laser pulse and Ti:sapphire system (680–1080 nm, 80 MHz, 140 fs, Chameleon II) as the light source. All measurements were carried out in air at room temperature. Two-photon absorption cross-sections were measured using two-photon-induced fluorescence measurement technique. The two-photon absorption cross-sections (δ) were determined in DMSO with 1 mM **MQ** by comparing their TPEF to that of fluorescein according to the literature [12].

2.3. Cell culture and two-photon fluorescence microscopy imaging

For two-photon bio-imaging, 293FT cells were cultured in DMEM supplemented with 10% FCS, penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Cytotoxicity assays show that **MQ** is safe enough for two-photon bio-imaging at low concentrations, and 293FT cells were cultured and stained with **MQ** (10 μM) within 30 min and washed by PBS buffer and then treated with Cys (100 μM) for another 30 min. Cells imaging was carried out on a confocal microscope (Zeiss LSM 510 Meta NLO). Two-photon fluorescence microscopy images of labeled cells were obtained by exciting the probe with a mode-locked titanium–sapphire laser source set at wavelength 710 nm.

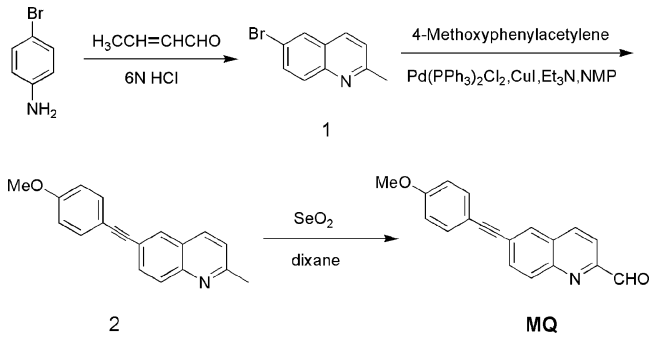
3. Results and discussion

3.1. Synthesis of *MQ*

As shown in Scheme 2, **MQ** can be easily synthesized in a three-step process with an overall yield of 61.2% from cheap materials [10b]. The structures of **MQ** and the intermediates were all confirmed by ^{1}H NMR and ^{13}C NMR.

3.2. UV–vis and fluorescence spectra responses

All spectroscopic measurements were carried out under simulated physiological conditions in pH 7.4 PBS/DMSO (1:9). As shown in Fig. 1, the UV–vis absorption spectra of **MQ** established a maximum absorption at 350 nm. Upon the addition of Cys, the maximum absorption was gradually shifted to 323 nm with an isosbestic point



Scheme 2. Synthesis route of **MQ**.

at 333 nm. The ratio of the absorbance ($A_{320\,nm}/A_{350\,nm}$) increased from 0.84 to 1.43 with the addition of Cys. The time-dependent test revealed that the reaction between **MQ** and Cys was finished in about 6 min (Fig. 1 inset and Fig. S1). The reaction was faster than previous results [9,13]. The quicker response of **MQ** to Cys will guarantee its application in the cell imaging.

Upon the addition of Cys, the maximum emission of **MQ** shifted from 505 nm to 410 nm (Fig. 2). Compared with previously reported probes for Cys [14], the blue-shift of fluorescent spectra is larger and can provide better ratiometric fluorescent detecting signal. The ratio of fluorescent intensity ($I_{410\,nm}/I_{505\,nm}$) increased from 0.28 to 2.70 on the addition of Cys and exhibited a linear relationship with Cys concentrations ranging from 0 to 2.4 equivalents (Fig. 2 inset). The detection limit was calculated to be 0.5 μM based on a signal-to-noise value of 3. These results suggested that **MQ** could afford the quantitative determination of Cys *in vitro*. The blue-shift of UV–vis and fluorescence spectra indicates the formation of thiazolidine and the switch-off of the ICT process in the system. The fluorescence quantum yields of **MQ** and **MQ** + Cys were determined to be 0.12 and 0.04 respectively. The fluorescence was quenched by the PET process from the nitrogen atom to the quinoline core (Scheme 1). Similar spectra changes were found for Hcy (Fig. S2).

3.3. Amino acids selectivity and pH stability

The optical responses of **MQ** to various amino acids were investigated by the emission spectroscopy. As shown in Fig. 3, none of these amino acids except Hcy and Cys induced the change of the fluorescence. Moreover, a remarkable color change of fluorescence

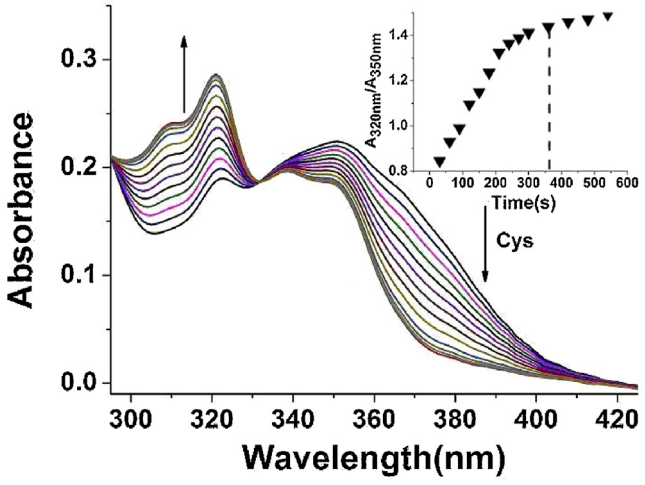


Fig. 1. Time-dependent (0–560 s) UV–vis absorption response of **MQ** (25 μM) in pH 7.4 PBS/DMSO (1:9, v/v) toward Cys (100 μM). Each spectrum was taken every 30 s. Inset: absorbance ratio ($A_{320\,nm}/A_{350\,nm}$) as a function of reaction time.