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Flavone-Based ESIPT Ratiometric Chemodosimeter for Detection of Cysteine in Living Cells

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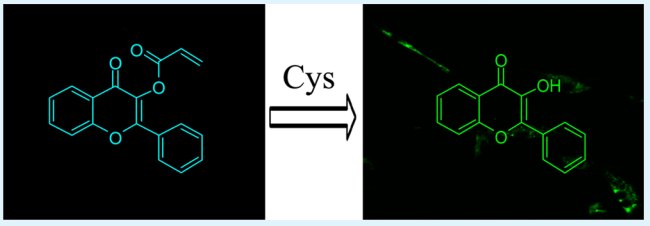
Supporting Information

ABSTRACT: We have designed and synthesized a novel ratiometric fluorescent chemodosimeter **MHF**-based ESIPT process for specific detection of cysteine among the biological thiols. The probe **MHF** shows very weak blue fluorescence under UV excitation. Upon addition of cysteine (Cys), the reaction of Cys with **MHF** induces acrylate hydrolysis, thereby enabling the ESIPT process to shift the weak blue emission to a strong green emission with about 20-fold enhancement. We utilized ^{1}H NMR spectra to elucidate the fluorescence sensing mechanism. Moreover, the cellular imaging experiment indicated the **MHF** possessed excellent selectivity, low cytotoxicity, and desirable cell permeability for biological applications.



KEYWORDS: *chemodosimeter, fluorescence, cysteine, ratiometric, selectivity, bioimaging*

INTRODUCTION

As one of the most important biological thiols, cysteine (Cys) plays a pivotal role in many biological processes such as reversible redox reaction and cellular detoxification and metabolism.[1,2] Low Cys levels could be related to many health issues such as hematopoiesis reduction, hair depigmentation, skin lesion development, and cancer,[3−5] as Cys is involved in the chemical regulation of many biological processes. A number of analytical methods for the detection of Cys have been developed using high-performance liquid chromatography (HPLC),[6] capillary electrophoresis,[7] electrochemical assay,[8] UV/Vis,[9] FTIR,[10] mass,[11] and fluorescence spectroscopy.[12,13] Among these methods, fluorescence probes are more desirable due to its high selectivity, low detection limit, fast response and great potential for bioimaging.[14−16]

Current fluorescent probes for Cys often utilize the nucleophilicity of thiol group in the sensing scheme.[17−35] However, lots of reported probes suffer from low selectivity, poor cellular uptake, interference of autofluorescence, and high cytotoxicity, which significantly limits their biological applications. Therefore, developing highly biocompatible and selective fluorescent probes to monitor the Cys levels is of great scientific interest, which requires the integration of the Cys' unique reactivity with a selective and reliable chemical event.

Recently, the fluorescent dyes based on excited-state intramolecular proton transfer (ESIPT) process, as seen from 2-(2′-hydroxyphenyl)benzoxazole, 1-aminoanthraquinone, and flavone, have been used as an attractive fluorescent signal transducer in sensors.[36−44] In comparison with the other fluorescent processes, such as electron transfer, ESIPT process can occur at a much faster rate ranging from fractions of picoseconds to tens of picoseconds.[45] Moreover, ESIPT dyes generally have large Stokes' shift (>150 nm), which minimizes the self-absorption and reduce the interference from autofluorescence for in vivo application.[46] Although the unique photophysical properties are known for decades, only one ESIPT sensor has been designed for Cys detection.[26]

Among ESIPT dyes, flavone dyes are a broad class of natural products, and have been extensively studied for their antioxidant properties and anticancer activities in the food and health sciences.[47] However, few flavone-based biosensors have been studied for bioimaging application (Scheme S2 in the Supporting Information). Herein, we present a novel flavone-based ratiometric fluorescence probe, 4-oxo-2-phenyl-4H-chromen-3-yl acrylate (**MHF**), which gives ESIPT emission upon binding cysteine in living cells (Scheme 1a). The sensor design utilizes both thiol and amino groups of Cys in a nucleophilic addition and subsequent cyclization reaction, in order to achieve specific recognition of Cys. Other prominent features of **MHF** include: (1) large emission spectral shift (from weak 380 nm to strong 510 nm) in responding to Cys, as a consequence of the ESIPT turn-on;[48] (2) linear response to Cys; (3) the flavone-based dye is of low cytotoxicity, good cellular uptake, which are desirable for medicinal biology and diagnostic applications.

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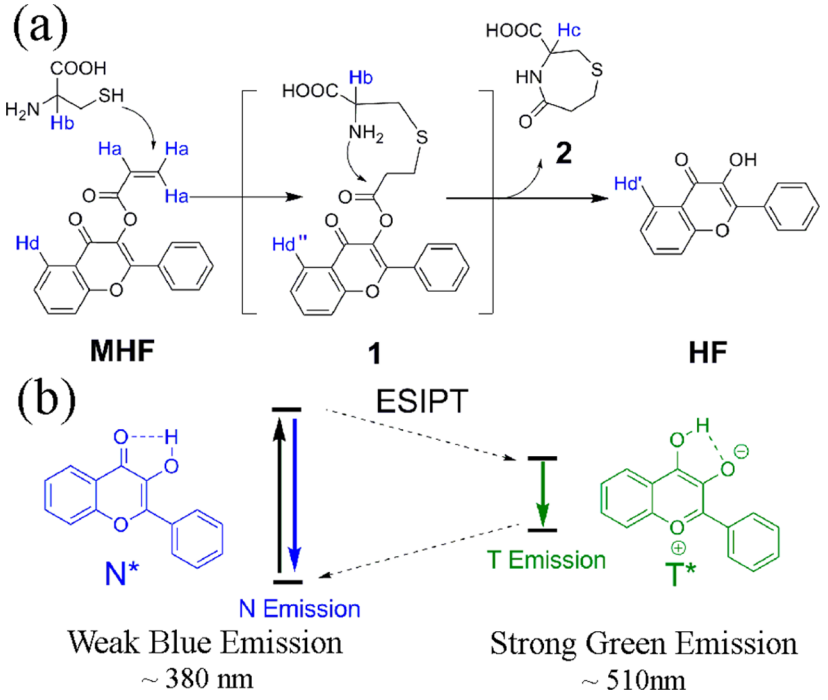
Scheme 1. (a) Chemical Structures of MHF along with the Proposed Sensing Mechanism; (b) Schematic Representation of ESIPT Process of HF



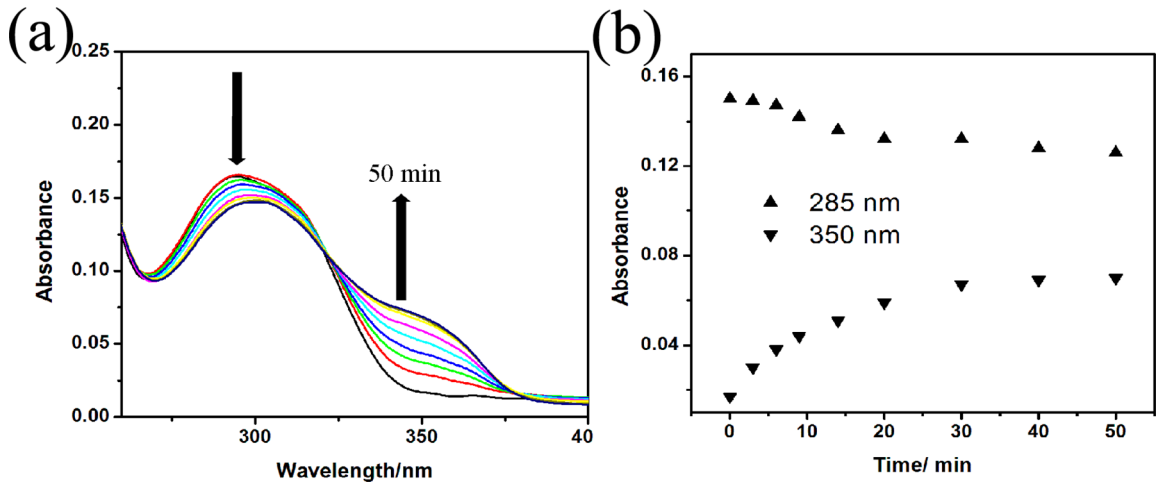


Figure 1. Time-dependent (a) absorption spectral changes and (b) absorbance changes (λ = 285 nm and 350 nm) of MHF (10μM) in the present of 100 μM Cys in MeCN-H_2O (1:1, v/v) solution with 10 mM HEPES buffer.

EXPERIMENTAL SECTION

Reagents and Instrumentation. ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker AVANCE II. UV−vis spectra were acquired on a Hewlett-Packard 8453 diode-array spectrometer. Fluorescence spectra were measured by RF-5301PC spectrometer The fluorescence quantum yields were obtained using quinine sulfate as the standard (Φ_{fl} = 0.53, 0.1 M H_2SO_4). Electrospray ionization (ESI) mass spectra were acquired with a Waters Synapt HDMS quadrupole/time-of-flight (Q/ToF) mass spectrometer. All the solvents for the fluorescence experiments were analytic grade, which were purchased from Fisher Scientific and used without further purification. 1 mmol/L **HF** and **MHF** were dissolved in MeCN as stock solutions and 10 mM biologically relevant analytes (Cys, Hcy, GSH, NaSH, Ala, Arg, Asn, Asp, Gln, Gluc, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Tau, Thr, Trp, Tyr, Val) were separately dissolved in distilled water. 10 mM HEPES solution was prepared as buffer solution. All UV/Vis and fluorescence titration experiments were performed using 10 μM of HF or MHF in 50% MeCN aqueous solution (pH 7.4, 10 mM PBS buffer) with varying concentrations of analytes at room temperature with 1 h reaction time. The cell imaging was obtained by X-Cite Series 120Q fluorescence microscopy. The blue channel filter: excitation 365 nm, beam splitter FT 395 nm, emission 445/50 nm. The green channel filter: excitation 450−490 nm, beam splitter FT 510 nm, emission 515-565 nm.

Synthesis of 4-Oxo-2-phenyl-4H-chromen-3-yl acrylate (MHF). 1 mmol **HF** and 1.2 mmol K_2CO_3 was dissolved in 20 mL of dry acetone in ice-water bath then 1.2 mmol acryloyl chloride in 10 mL id acetone was slowly added into the solution. The mixture was stirred for 12 h then the solvent was removed in reduced pressure. The crude product was purified by column chromatography on silica gel. Yield = 52%. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 6.04−6.07 (d, 1H), 6.33−6.42 (m, 1H,), 6.61-6.67 (d, 1H), 7.26-7.45 (t, 1H), 7.47−7.56 (m, 3H), 7.59-7.61 (d, 1H), 7.71−7.76 (t, 1H), 7.87−7.90 (dd, 2H), 8.27-8.30 (d, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 118.1, 123.7, 125.3, 126.2, 126.9, 128.3, 128.7, 130.0, 131.3, 133.7, 134.0, 155.7,

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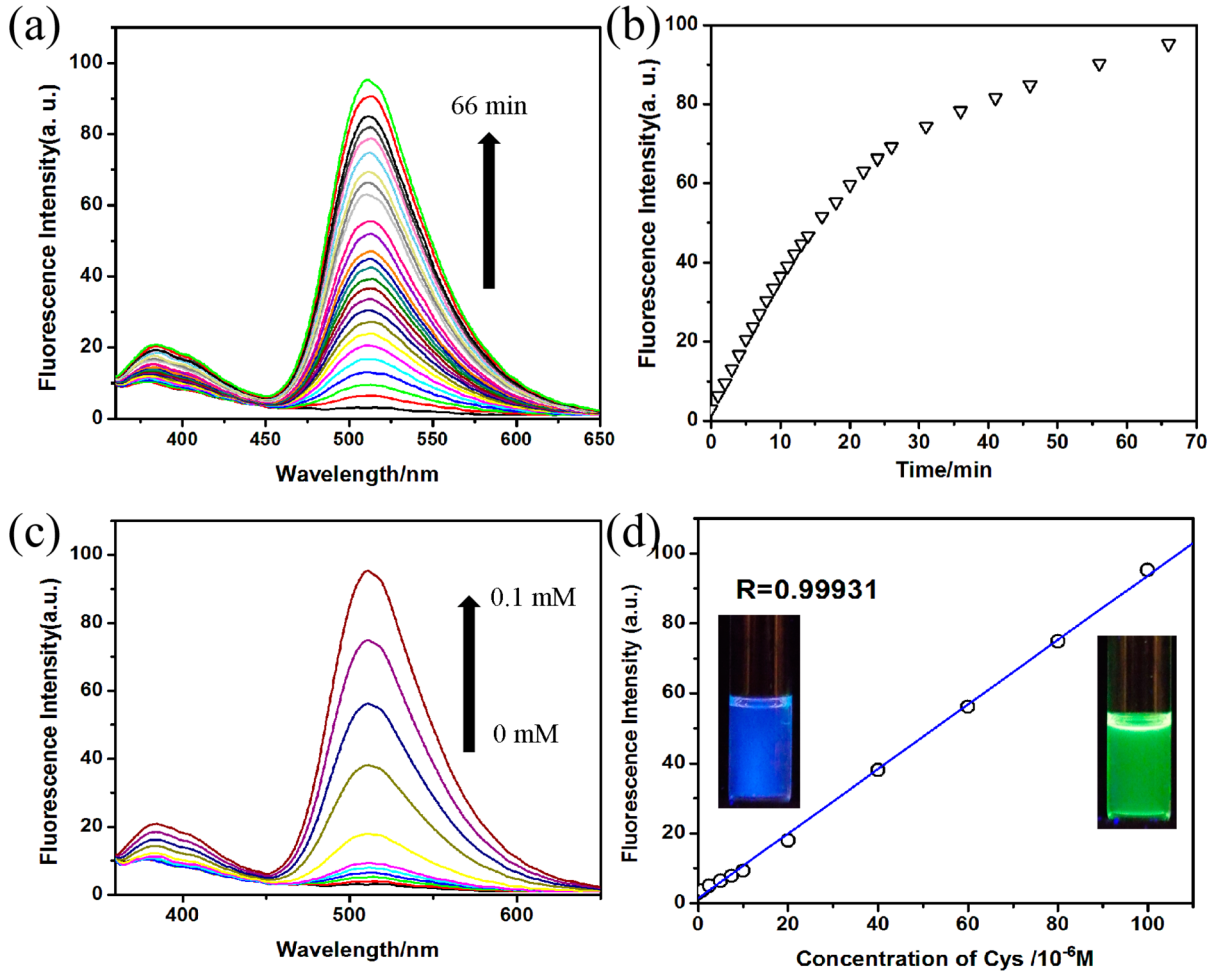

Figure 2. Time-dependent (a) fluorescence spectral changes and (b) fluorescence intensities (λ=510 nm) of **MHF** (10 μM) in the present of 100 μM Cys in MeCN-H_2O (1:1, v/v) solution with 10 mM HEPES buffer. (c) Fluorescence spectra changes and (d) fluorescence intensity changes (λ=510 nm) of 10 μM **MHF** in the presence of increasing concentrations of Cys (final concentration: 0, 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1 mM) in MeCN-H_2O (1:1, v/v) solution with 10 mM HEPES buffer. Each spectrum was recorded after 60 min.

156.4, 163.1, 172.1. HRMS: *m*/*z* calcd for $C_{18}H_{14}O_5$ $(M + Na)^+$, 315.0633; found, 315.0616.

Cell Culture. Human mesenchymal stem cells (hMSCs) (Lonza, Walkersville, MD) were cultured in serum-containing MSCBM medium (Lonza) supplemented with MSCGM SingleQuots (Lonza) according to manufacturer's specifications. hMSCs (Passage 5) were seeded at a density of 5.0×10^4 cell/cm^2. Before treatment of **MHF**, the control cells were incubated with media containing 100 μM NEM for 30 min at 37 °C to react with cellular thiols. The cells were then briefly washed with 1 mL of PBS. After incubation with 20 μM **MHF** (1% DMSO) for 1 h at 37°C, fluorescence images were taken using a fluorescence microscope. The cytotoxicity of the **MHF** towards stem cells was determined by conventional MTT assays.

RESULTS AND DISCUSSION

MHF was conveniently synthesized from acylation of 3-hydroxyflavone (**HF**) with acryloyl chloride. **MHF** exhibited one absorption peak at 290 nm, while **HF** had two absorption peaks at 310 and 340 nm (see Figure S1 in Supporting Information). When being excited at 350 nm, **MHF** gave a weak emission peak at ~380 nm. Addition of Cys to **MHF**, however, gave two emission bands at 380 and 510 nm, which can be attributed to the normal isomer (N* emission) and tautomer (T* emission) of **HF**, respectively (see Scheme 1b). Observation of the intense green emission from "**MHF** + Cys"

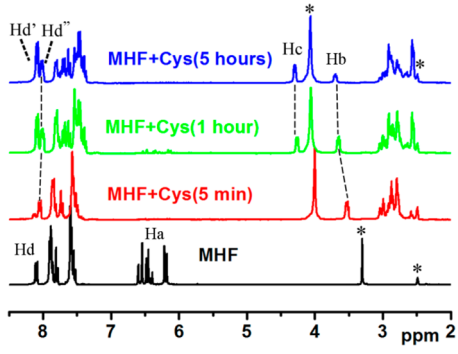

Figure 3. ^{1}H NMR spectrum of **MHF** in *d*6-DMSO, and the resulting spectrum after addition of 1 equiv. Cys in D_2O for 5 min, 1 h, and 5 h. The starred "*" signals are attributed to DMSO and water solvents.

sample indicated the formation of **HF**, as the reaction of Cys with **MHF** released the hydroxyl group in flavone, thereby enabling the ESIPT process to shift the emission signal to a longer wavelength. The new emission peak can be used for the ratiometric fluorescent measurement, as the ratio of two fluorescent bands (instead of the absolute intensity of one band) can determine the analytes more accurately with the minimization of the background signal.[49]

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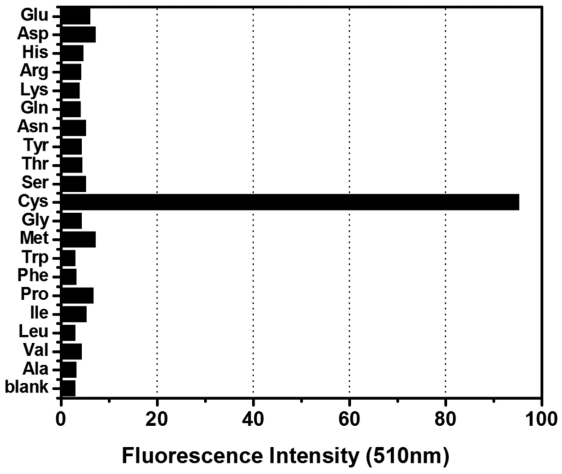

Figure 4. The fluorescence intensities (λ = 510 nm) of 10 μM **MHF** upon addition of 100 μM physiological important amino acids (Glu, Asp, His, Arg, Lys, Gln, Asn, Tyr, Thr, Ser, Cys, Gly, Met, Trp, Phe, Pro, Ile, Leu, Val, and Ala) in MeCN-H_2O (1:1, v/v) solution with 10 mM HEPES buffer.

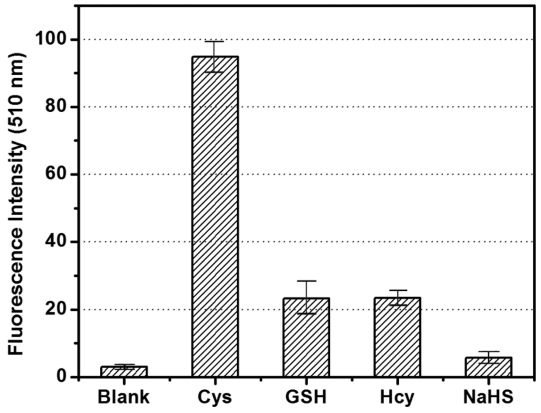

Figure 5. The fluorescence intensities (λ = 510 nm) of 10 μM **MHF** upon addition of 100 μM biologically important thiols (Cys, GSH, Hcy, and NaHS) in MeCN-H_2O (1:1, v/v) solution with 10 mM HEPES buffer.

The optical sensing behavior of **MHF** toward Cys was investigated by using a 10 μM **MHF** in MeCN-H_2O (1:1, v/v) solution (pH 7.4, 10 mM PBS buffer). Upon addition of 100 μM Cys to the solution of **MHF**, the absorption band at around 350 increased gradually over time, meanwhile the band at 290 nm decreased with a 10 nm red shift, shown in Figure 1. For the fluorescence spectra, the addition of Cys caused an apparent ratiometric fluorescence response. The N* emission slowly increased and became doubled after one hour reaction, whereas a significantly higher fluorescence (>20-fold) was observed from the tautomer (T* emission), as seen in Figure 2a, b. To verify this mechanism of the Cys-induced acrylate cyclization,[50] we examined the ^{1}H NMR of **MHF** in *d*6-DMSO at room temperature. As seen in Figure 3, after addition of 1 equiv. of Cys in D_2O for 5 min, the characteristic alkenyl proton H_a (labeled in the structure in scheme 1a) from 6 to 7 ppm disappeared completely, suggesting a very fast reaction between thiol and alkene, which produced intermediate **1**. However, the lactam proton H_c at ~4.3 ppm in **2** wasn't found, which indicated the cyclization was relatively slow. As the reaction proceeds, the intensity of H_c became relatively higher than the intensity of methine proton H_b in Cys and **1** at ~3.7 ppm. The proton signal $H_{d'}$ in **HF** was gradually increased, along with the decrease in proton signals H_b and $H_{d''}$ in **1**. The reaction over 10 h at room temperature (seevFigure S2 in the Supporting Information) showed that the reaction sequence in Scheme 1a proceeded cleanly, making the process reliable for Cys detection.

The sensitivity of **MHF** was studied by fluorescence response towards various concentrations of cysteine. Panels c and d in Figure 2 showed that with the increase of Cys concentration, the fluorescence intensity at 510 nm was enhanced dramatically. The fluorescence intensity at λ = 510 nm was linearly proportional to the amount of Cys ranging from 10 μM to 100 μM with a detection limit of lower than 1 μM. Cys concentration normally range from 16.5 to 33.0 μM in healthy individual urine.[51] Because 10 μM of Cys could enhance T* emission intensity of **MHF** for about 2-fold in pure water, this probe could be used for early detection of Cys-related metabolism disease (see Figure S3 in the Supporting Information). The selectivity of chemodosimeter **MHF** towards various physiological important amino acids and biological

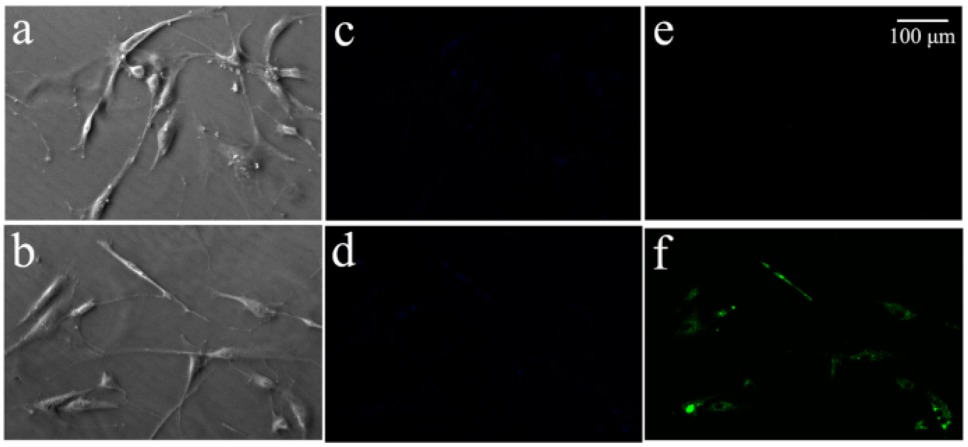

Figure 6. Fluorescence microscopy images of hMSCs. (a) Bright-field image, and fluorescence images in (c) blue and (e) green channel after hMSCs were pre-treated with 100 μM NEM for 30 min, and then incubated with 20 μM **MHF** for 1 h. (b) Bright-field image, fluorescence images in (d) blue and (f) green channel after hMSCs being incubated with 20 μM **MHF** for 1 h at 37°C.

thiols was also investigated. As showed in Figure 4 and Figure 5, **MHF** showed highly selective for Cys with remarkable fluorescence intensity enhancement, only mercapto species such as Hcy and GSH showed slight interference. Therefore, MHF could be a practically useful probe for effective recognition of Cys.

To further investigate the biological application of **MHF**, the fluorescence microscopy experiment was carried out. When human mesenchymal stem cells (hMSCs) were incubated with 20 μM **MHF** in culture medium at 37 °C for 1 h, relatively weak blue N* emission (Figure 6d) but strong green T* emission (Figure 6f) were observed, which was attributed to the formation of **HF** via Cys-induced acrylate hydrolysis and indicated a very good cellular uptake. A control experiment was performed to verify that the sensor's green fluorescence was attributed to the reaction with Cys. Thus, when hMSCs were pretreated with 100 μM NEM (N-ethylmaleimide, an efficient thio-reactive compound) for 30 min, and then incubated with 20 μM **MHF** in culture medium at 37°C for 1 h, the green emission was very weak (Figure 6e), because Cys were consumed by NEM. Moreover, **MHF** exhibited very low cytotoxicity towards hMSCs, which was evaluated by means of MTT assays,[52] after the cells were incubated for 24 h in the presence of 50 μM **MHF** (see Figure S4 in the Supporting Information).

CONCLUSION

In summary, we have developed a novel ratiometric chemodosimeter **MHF** by using the low-cytotoxic flavonoid dye and ESIPT turn-on. The **MHF** exhibited high sensitivity for Cys (detection limit 1 μM) whose excellent selectivity differentiates it not only from the essential amino acids but also from the biologically important thiols. The probe was successfully used for fluorescent imaging of intracellular Cys, demonstrating its potential for a broader range of biological sample analysis.

ASSOCIATED CONTENT

Supporting Information

Experimental details for synthesis of MHF, its ^{1}H and ^{13}C NMR data, and additional absorption and fluorescence spectra for MHF and HF. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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