A Novel Ratiometric Two-Photon Fluorescent Probe for the Detection of Biothiols in Solution and Imaging of Living Cells

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A novel fluorescent probe for selective detection of thiols in acidic solutions and labeling of acidic organelles in live cells†

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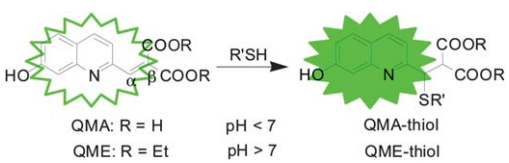
A novel fluorescent probe, quinoline α,β-unsaturated diacid (QMA), as compared to its ester QME, was constructed and can selectively detect thiols in acidic solutions (pH < 7) *via* a H-bond activated Michael addition. Furthermore, labeling of lysosomes and cell imaging were achieved by the detection of biothiols in different domains of live cells.

Thiols are an important class of molecules in biological systems and chemical science. Several aliphatic thiols including cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) play essential roles in many physiological processes.[1] For example, GSH is critical in maintaining redox homeostasis,[2] which is important for maintenance of cellular defense against reactive oxygen species and for a number of biological processes. Abnormal levels of cellular thiols are associated with many human diseases, such as slow growth, hair depigmentation, edema, lethargy, liver damage, loss of muscle and fat, skin lesions and weakness,[3] cardiovascular diseases and Alzheimer's disease.[4] Therefore, selective detection of cellular biothiols is of growing importance.

Optical approaches based on synthetic colorimetric and fluorescent molecular probes have attracted increasing interest due to their simplicity, inexpensiveness, sensitivity and selectivity during the last decade.[5] A variety of colorimetric and fluorescent probes for thiols have been constructed by exploiting the high nucleophilic reactivity or transition metal-affinity of the thiol group, which involve specific reactions between probes and thiols, such as Michael addition,[6] cleavage reactions by thiols,[7] cyclization with aldehyde,[8] metal complexes-displace coordination[9] and others,[10] in the past four years. Among these probes, the most cases involve in the electrophilic reactivity of probe molecules and the nucleophilicity of thiols, such as the probes *via* the Michael addition or the aromatic nucleophilic substitution (S_NAr). Because the deprotonated thiolate anion is more nucleophilic than its neutral form, the nucleophilicity of thiols depends on the pK_a values (*ca.* 6–9) of thiol groups. Hence, most fluorescent probes were designed to detect thiols in slightly alkaline solutions (pH 7.2–9), which is the physiological condition, while the probes to detect thiols at low pH (<6) have rarely been reported.[11] Herein, we report a novel florescent probe, quinolin 2-methenyl malonic acid (QMA), which can react with thiols only in acidic solutions (pH < 7). In contrast, its ester QME displays a contrary pH effect on the sensing reactivity, which can act only at pH > 7, like most Michael-type thiol probes reported,[6] shown in Scheme 1. Furthermore, this difference was revealed in fluorescence images of living cells by confocal laser scanning microscopy.

A synthetic procedure of probe molecules is an easy four-step route with 7-hydroxy-2-methyl-quinoline as the starting material, as shown in Scheme 2. There are two key steps: (i) the methyl group in **1** is oxidized with SeO_2 to form the aldehyde group in **2**,[12] (ii) 2-carbaldehyde **3** reacts with malonic acid or its ethyl ester *via* Knoevenagel condensation[13] to give target compounds, QMA and QME.

The two target compounds emit very weak fluorescence, and their 7-ethoxy analogues give intense fluorescence. Hence, 7-hydroxy groups in QMA and QME play a key role in their low fluorescence efficiency. A nonradiative process, deprotonation of 7-hydroxy and subsequent intramolecular electron transfer from the hydroxylate group to the positively charged pyrimidinium ring, is a minor pathway for inactivation of excited 7-hydroxyquinoline with a high



Scheme 1 Two fluorescent probes that respond to thiols in different pH ranges of solutions.

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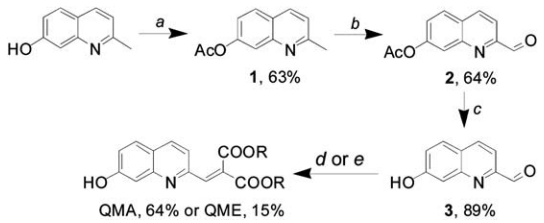


Scheme 2 Synthesis of probe molecules: (a) 4-dimethylaminopryidine, CH_2Cl_2, r.t., 4 h; (b) SeO_2, dioxane, reflux, 12 h; (c) CH_3ONa, methanol, r.t., 30 min; (d) malonic acid, piperidine, ethanol, 50 °C, 30 min; (e) malonic acid diethyl ester, piperidine, ethanol, 50 °C, overnight.

fluorescence efficiency.[14] The nonradiative process may be enhanced when the electron-withdrawing moiety, 2-methenyl malonic acid or ester linked to the quinoline, leading to a weak fluorescence. After the Michael addition of thiols to the α,β-double bond of QMA and QME, the fluorescence of 7-hydroxyquinoline could be restored to display fluorescence turn-on responses.

As expected, when 5 equiv. of Cys was added into QMA acidic solution of DMSO–0.1 M phosphate-critic acid buffer (v/v 2 : 98, pH 3.8), its time-dependent UV-Vis absorption spectra exhibit a ratiometric change with isosbestic points at 350 nm and 362 nm, and the fluorescence gradually increases to reach a maximum in 40 min (Fig. 1). Based on time-dependent absorption spectra, the second-order rate constant of this sensing reaction was obtained, k = 5.4 M^{-1} s^{-1} at room temperature, and 5.8 M^{-1} s^{-1} for Hcy and 4.2 M^{-1} s^{-1} for GSH under the same conditions. These rate constants are much higher than those of probes reported.[6k,6o,6s]

The sensing reaction as a Michael addition was confirmed by ^{1}H NMR spectroscopy. Upon the addition of Cys, the vinylic proton (H^α at 7.66 ppm) of QMA disappears, with the concomitant appearance of new peaks around 4.39 and 4.66 ppm, which are assigned to two protons of the adduct product QMA–Cys (Fig. 2). In addition, HPLC chromatograms show that the Michael reaction between QMA and Cys is a single effective process (Fig. S1, ESI†). Mass spectral analysis of the resulting mixture provides an additional support for the Michael reaction, that is, a peak, m/z 379.0603, which well matches with the anion of the adduct, $C_{16}H_{15}N_2O_7S$ (calcd 379.0605 [M − H^+]), in the mass spectrum from the reaction mixture of QMA and Cys·HCl in DMSO (Fig. S2, ESI†).

The fluorescence response of QMA (20 μM) for different amounts of Cys was observed in the acidic solution (pH 3.8) (Fig. 3). When the concentration of Cys was increased to 5 equiv., no further increase in fluorescence intensity was observed. The fluorescence alternation displays a well linear relationship with the concentration of Cys in the range from 0 to 3 equiv. This implies that a quantitative analysis of thiols can be achieved in this concentration range.

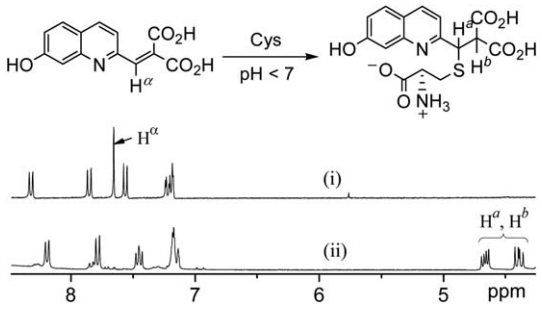


Fig. 2 Partial ^{1}H NMR spectra of QMA (15 mM) before (i) and after (ii) addition of Cys·HCl (1.5 equiv.) in DMSO-d_6 at room temperature.

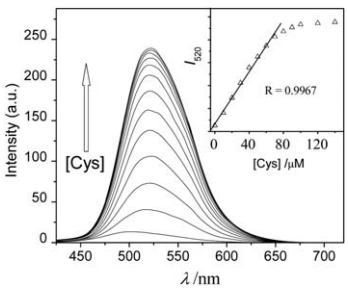


Fig. 3 Fluorescence spectra of QMA (20 μM) in the acidic solution (pH 3.8) in the presence of different concentrations of Cys. Inset: fluorescence maximum as a function of Cys concentration, and a straight line obtained by linear fitting of maximum *vs.* [Cys] from 0 to 70 μM.

To investigate pH effects on the sensing reactivity of QMA, we monitored the reaction of 20 μM QMA with 5 equiv. of Cys by UV-vis absorption spectroscopy in various pH solutions ranging from pH 3 to 10. The observed rate constants (k_{obs}) were obtained from time-dependent UV-vis absorption spectra, as shown in Fig. 4, left. The plot clearly shows that the Michael addition can occur only in acidic solution (pH < 7). In contrast, the corresponding plot for QME exhibits a contrary pH effect, working in alkaline solutions (pH > 7). The fluorescence enhancements (F/F_0) in various pH solutions reveal a similar pattern to their k_{obs} (Fig. 4, right).

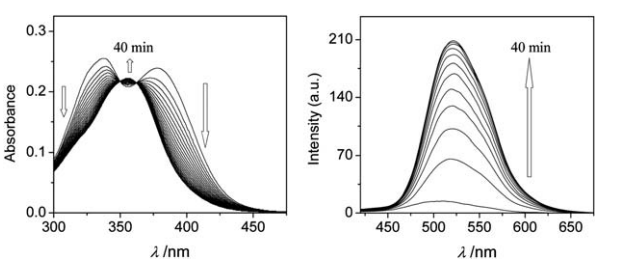


Fig. 1 UV/vis absorption (left) and fluorescence (right, λ_{ex} 360 nm) spectra of QMA (20 μM) in the acidic solution (pH 3.8) in the presence of 5 equiv. Cys recorded at certain time intervals from 0 to 40 min.

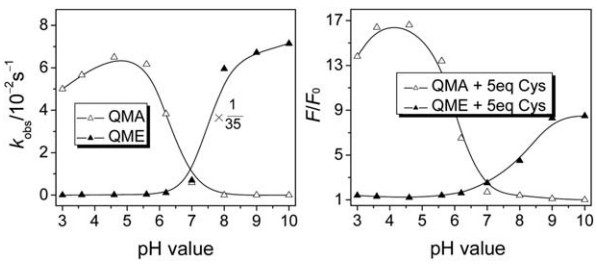


Fig. 4 Left: observed rate constants of the Michael addition of QMA or QME with 5 equiv. of Cys in various pH solutions. Right: fluorescence enhancements of QMA or QME after being treated with 5 equiv. of Cys for 30 min as a function of pH values.

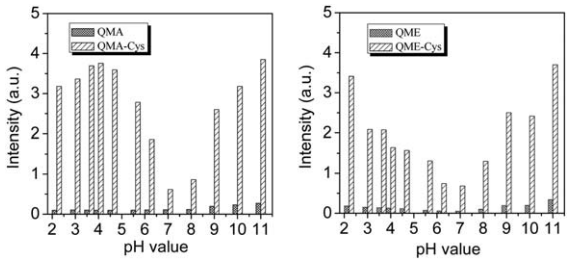


Fig. 5 Fluorescence maxima of QMA and its adduct QMA–Cys (left), and QME and its adduct QME–Cys (right) in various pH solutions, λ_{ex} 360 nm.

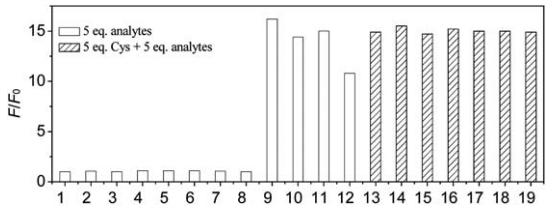


Fig. 6 Fluorescence enhancements of QMA solutions (20 μM, pH 3.8) with or without various analytes (5 equiv.): 1, blank; 2, aniline; 3, phenol; 4, urea; 5, Gly; 6, Met; 7, Ser; 8, Asn; 9, MAA; 10, Cys; 11, Hcy; 12, GSH; 13, Cys + aniline; 14, Cys + phenol; 15, Cys + urea; 16, Cys + Gly; 17, Cys + Met; 18, Cys + Ser; 19, Cys + Asn.

Furthermore, fluorescence maxima of QMA, QME and their adducts with Cys in various pH solutions ranging from 3 to 7 were determined, as shown in Fig. 5. Both QMA and QME emit weak fluorescence in the pH range, and their adducts, QMA–Cys and QME–Cys, emit strong fluorescence in the pH range except around 7. Therefore, two probe molecules can effectively react with thiols in different pH ranges, *i.e.*, pH < 7 for QMA and pH > 7 for QME, and display fluorescence turn-on responses. The sensing behavior of QME is similar to that of probes reported,[6,7] which are usually not reactive at pH < 7 due to thiols in neutral forms. In general, if a probe is reactive in acidic solutions, it may lose selectivity for thiols over other nucleophiles. In contrast, QMA reacts with thiols only in acidic solutions, and its adduct QMA–Cys gives large fluorescent enhancements in acidic solutions.

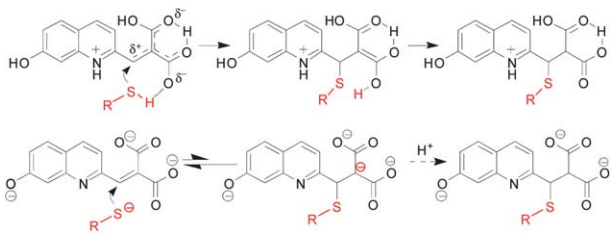
On the basis of their molecular structures, the new sensing behavior of QMA should be derived from its different ionization states of two carboxyl groups. The pK_a values of two carboxyls of two QMA analogues were obtained to be about 3.2 and 6.0.[15] Thus, in acidic solutions, there is an intramolecular hydrogen bond (H-bond) between two carboxyl groups of QMA, and two carboxyl groups would be coplanar with the α,β-unsaturated bond, in which C^{α} displays electrophilicity (Scheme 3, top) based on its resonance structures. The RS–H bond could be activated by an intermolecular H-bond formed with a carboxyl group, thereby, the nucleophilic RS moiety attacks the C^{α}, undergoing the Michael addition.

Solvent effects on the reactivity of QMA with mercaptoacetic acid (MAA) in three solvents provide a further support for the H-bond assisted Michael reaction. The observed rate constants (k_{obs}) show that in two aprotic solvents, dioxane (0.44 min^{-1}) and DMSO (1.2 min^{-1}), the reaction is much faster than that in the protic solvent, the acidic buffer (pH 3.8) (0.14 min^{-1}) (Fig. S3, ESI†).

However, in alkaline solutions, the nucleophilic addition would be suppressed because of electrostatic repulsion between QMA as a carboxylate anion and thiolate anion. More importantly, the intermediate from the nucleophilic addition may be unstable and undergo a bond cleavage back to starting materials (Scheme 3, bottom). Fig. 5 shows that the adduct QMA–Cys resulting from the protonation of the intermediate is stable in alkaline solutions (pH 7–11), but the protonation could not occur due to a short life-time of the unstable intermediate and a low proton H^+ concentration in alkaline solutions. Most fluorescent probes for aliphatic thiols cannot discriminate thiophenols and aliphatic thiols because thiophenols are more active. However, a control experiment showed that the reaction of QMA with thiophenol was not detected in alkaline solutions such as pH 9.

Next, the specificity of QMA toward thiols was investigated by fluorescence response of QMA in the presence of various biologically relevant analytes. No fluorescence enhancement was observed from QMA solutions after the addition of 5 equiv. of aniline, phenol, urea and other representative amino acids (Gly, Met, Ser and Asn), respectively (Fig. 6). However, under the same conditions, QMA exhibits a larger fluorescence response to MAA, Cys, Hcy and GHS as well as to Cys under the potential competition of nucleophiles and biologically relevant analytes. In addition, no response to amino acids except Cys can be observed by naked eyes from the color and the green emission of solutions (Fig. 7). These results show the specific response of QMA to thiols.

To test the capability of two probes QMA and QME to image thiols in living cells, MDA-MB-231 cells were seeded on the coverslips in 24-well plates at a density of 50 000 cells per well and incubated at 37 °C. DMSO solutions of QMA and QME were added respectively to a well to give a concentration of 20 μM (DMSO/culture medium, v/v 1/1000). After being incubated for 3 h, cells were further stained with LysoTracker® Red, which can label acidic



Scheme 3 Top: a proposed H-bond assisted Michael reaction of QMA in acidic solutions; bottom: addition of a thiolate anion to QMA in alkaline solutions.

Fig. 7 Photograph for the color change (top) and fluorescence (bottom) of QMA (20 μM) solutions in the presence of various amino acids (5 equiv.) in the buffer solution (pH 3.8).

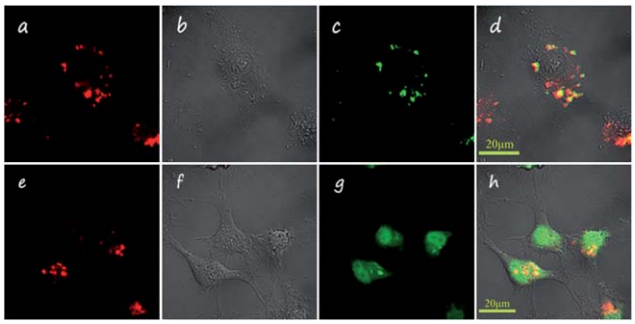


Fig. 8 Confocal fluorescence images of the MDA-MB 231 cells treated simultaneously with 20 μM QMA (a–d) or QME (e–h) and LysoTracker® Red for staining of lysosomes. (a, e) Red fluorescence images of LysoTracker® Red, (b, f) brightfield images, (c, g) green fluorescence images of QMA and QME, and (d, h) overlap images of (a), (b) and (c) or (e), (f) and (g), respectively.

organelles such as lysosomes (pH ~ 5) of live cells in red fluorescence. The probe has several important features, including high selectivity for acidic organelles and effective labeling of live cells at nanomolar concentrations.[16] The fluorescence images of QMA show that only partial regions of cells emit green fluorescence, which well overlaps with red fluorescence of LysoTracker® Red (Fig. 8a–d). This implies that QMA reacts with biothiols only in acidic organelles. The cells stained with QME emit green fluorescence in almost whole cells (Fig. 8e–h). Therefore, QMA can react with biothiols to form the adduct with intense fluorescence in acidic domains (*e.g.* lysosome), and QME can react with biothiols in the slightly alkaline cytosol (pH 7.2), resulting in green emission, thereby, detecting biothiols in most cellular regions. These observations are in agreement with solution results.

In conclusion, we have developed a novel thiol fluorescent probe, which can work only in acidic solutions (pH < 7) *via* a Michael reaction with the thiol in its neutral form, whereas no response to a thiolate anion in alkaline solutions. The new sensing reaction carries out the unprecedented sensing mechanism *via* a H-bond activated Michael addition under acidic conditions. Its ester QME reveals a contrary pH effect on the sensing reactivity like most probes reported. Moreover, simultaneous staining of lysosomes by QMA and LysoTracker® Red yielding identical staining patterns shows that QMA could be employed to detect biothiols in acidic organelles, thereby, label lysosomes in live cells. QME can detect biothiols in the slightly alkaline cytosol, and achieve fluorescence images of live cells. Further investigation for the application of QMA remains for quantitating of the number of lysosomes by flow cytometry or fluorometry.

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