Talanta 116 (2013) 508–513

Contents lists available at ScienceDirect

Talanta

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

Fluorescent probes that distinguish proteins with single or two close mercapto groups

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ARTICLE INFO

Article history:
Received 21 May 2013
Received in revised form
13 July 2013
Accepted 16 July 2013
Available online 24 July 2013

Keywords:
Fluorescent probe
Mercapto group detection
Maleimide
Protein detection

ABSTRACT

Two water soluble fluorescent probes with quaternary ammonium salts as solubilization groups were designed and synthesized to detect proteins with close mercapto groups. The unique structure of V-shape oligo(o-phenylene-ethynylene) backbone connecting two maleimide groups as mercapto group recognition sites enables the probes to distinguish a single mercapto group from two close mercapto groups in proteins. The probe had no fluorescence response to proteins with only one mercapto group, but had strong fluorescence response to proteins with mercapto group combination at the concentration of 10^{-6} mol/L. The probe worked under a wide range of pH, from 4 to 8. This work also demonstrates the concept of functional group combination detection.

1. Introduction

Fluorescent probes have attracted great interest in recent years due to their high sensitivity, fast response and ease of measurement [1–3]. These fluorescent probes target different analytes, including explosives [4–7], heavy metal ions [8–13], pollutants [14–17], biomolecules [18–21] and so forth. A large part of these probes are based on a specific reaction of the target functional group, such as Michael addition for mercaptans [18,22–24], imine formation for aldehydes [16,25], or boronate esterification to polyols [26,27]. Certain functional groups are pivotal in some life processes and their detection is an important aspect in bioanalysis. For example, mercaptans play important roles in metabolism, catalysis, transport, and are related to the cell aging process [28,29]. Imbalance in mercaptans metabolism and abnormal mercaptan levels are detected in many diseases such as cancer and AIDS [30,31]. However, most biological processes require several functional groups working together. For instance, thioredoxin has two mercapto groups close to each other as the active site for the reduction–oxidation cycle [32,33]; while papain's peptide cleavage activity comes from a mercapto group and a nearby imidazole ring [34]. Therefore, fluorescent probes targeting a combination of functional groups are worth investigating, but up until now, these kinds of fluorescent probes are rarely reported and seldom applied in bioanalysis [35–37].

Herein, we present a novel fluorescent probe targeting two close mercapto groups in proteins. As shown in Scheme 1, we adopt a structure we used before [38] as a molecular ruler for dithiols and further modify it into two water soluble molecules Probe 1 and Probe 2. The oligo(o-phenylene-ethynylene) (OPE) backbone is the fluorescent core which connects the two maleimide groups serving as the mercapto group recognition sites. Maleimide can react with mercapto groups mildly through Michael addition reaction and switch the fluorescence on. Quaternary ammonium salts are introduced to provide water solubility. The two maleimide groups quench the fluorescence of the OPE backbone due to a low lying n,pi* transition associated with the maleimide ring [18]. Only when both maleimide groups react with mercaptans, the fluorescence will fully recover. The fluorescence will remain quenched if only one maleimide group is reacted. The rigid backbone fixes the two maleimide groups at the distance of about 1 nm, which defines the distance of the two targeting functional groups. And the backbone's V-shape also limits the possibility of one probe molecule reacting with two mercapto groups from two different analyte molecules, if the steric hindrance of the analyte molecules, for example a protein, is large enough. Therefore, the probe only responds to proteins with two close mercapto groups. Proteins with a single mercapto group will not be responded (Fig. 1). With this method, we would like to introduce the concept of functional group combination detection.

2. Experimental

2.1. Materials and equipments

Tetrahydrofuran (THF) and triethylamine (Et_3N) that were used in Sonogashira coupling reactions were distilled under N_2 protection over Na and CaH_2, respectively. All other reagents and solvents

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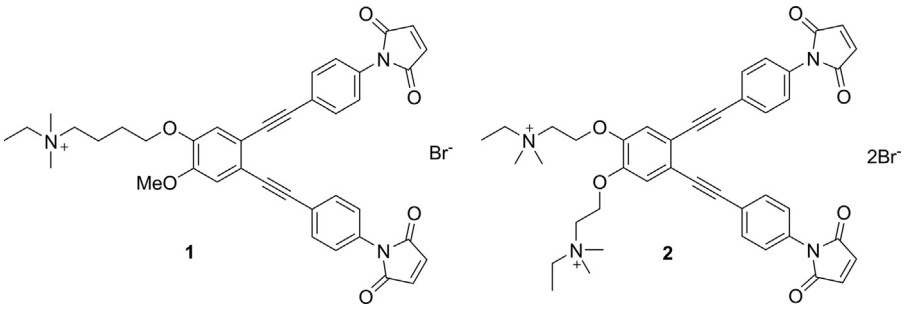
http://dx.doi.org/10.1016/j.talanta.2013.07.032



Scheme 1. Chemical structures of water soluble fluorescent probes, **Probe 1 and Probe 2**.

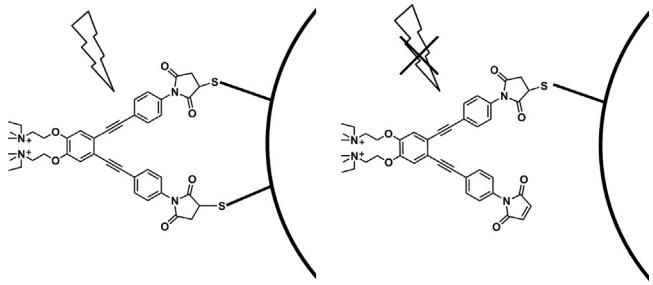


Fig. 1. A schematic illustration of how **Probe 2** responds to analyte molecules with a single (right) or two close (left) mercapto groups.

used in the syntheses were purchased from commercial suppliers and used without further purification. DMSO used in UV spectroscopy was purchased from commercial suppliers and used without further purification. Water was purified with a Milli-Q pure water system. Peptides **P1** and **P2** were synthesized by Shanghai Science Peptide Biological Technology Co., Ltd., with the purity above 75%. Bovine serum albumin (98%, pH: 6.5–7.2) was purchased from J&K Scientific Ltd. Metallothionein-1 (99%) was purchased from Dalian Free Trade Zone United Botai Bio-tech Co., Ltd. And it was stored as aqueous solution (2×10^{-4} mol/L) with the addition of tris(2-carboxyethyl)phosphine (2 mg/mL) to prevent oxidation.

^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 300 (300 MHz) spectrometer or a Bruker Avance 400 (400 MHz) spectrometer. Elemental analysis was performed using an Elementar VARIO EL elemental analyzer. ESI MS was performed with a Bruker Apex IV FTMS. Absorption spectra were recorded on a Perkin-Elmer Lambda 35 UV–vis spectrometer. Photoluminescence spectra were recorded on a Perkin-Elmer LS55 luminescence spectrometer.

2.2. Syntheses of fluorescent probes: *Probe 1*, *Probe 2* and *Probe 3*

4,5-Diiodo-2-methoxyphenol (**Compound 4**) [39], 1,2-bis-(2-p-tolylsulphonylethoxy)benzene (**Compound 8**) [40] and 4-ethynylaniline [41] were synthesized according to literature.

1-(4-Bromobutoxy)-4,5-diiodo-2-methoxybenzene (Compound 5): **Compound 4** (0.52 g, 1.4 mmol), 1,4-dibromobutane (1.0 mL, 8.3 mmol) and K_2CO_3 (0.40 g, 2.9 mmol) were dissolved in 5 mL of DMF and heated at 80 °C for 8 h. After the reaction mixture was cooled to room temperature, it was diluted with water and extracted with DCM. The organic phase was washed with water and dried over Na_2SO_4. After the solvent was removed, the residue was purified by flash column chromatography to obtain **Compound 5** as a white solid (0.62 g, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.24 (2H, s), 3.98 (2H, t, J=6.0 Hz), 3.81 (3H, s), 3.98 (2H, t, J=6.4 Hz), 2.10–1.92 (4H, m); ^{13}C NMR (100 MHz, $CDCl_3$): δ 150.0, 148.9, 123.3, 122.1, 96.4, 96.0, 68.3, 56.2, 33.2, 29.3, 27.6; anal. calcd for $C_{11}H_{13}BrI_2O_2$: C, 25.86; H, 2.56; found: C, 25.92; H, 2.60. EI-MS: calcd for $C_{11}H_{13}BrI_2O_2$: 509.8, 511.8; found: 510, 512.

Compound 6: Under N_2 protection, **Compound 5** (0.30 g, 0.59 mmol), 4-ethynylaniline (0.15 g, 1.28 mmol), $Pd(PPh_3)_2Cl_2$ (17 mg, 0.024 mmol) and CuI (7 mg, 0.037 mmol) were dissolved in 10 mL of THF and 3 mL of triethylamine and heated at 40 °C for 5 h. After the reaction mixture was cooled to room temperature, it was diluted with ethyl acetate and filtered. The solvent was removed from the filtrate and the residue was purified by flash column chromatography to obtain **Compound 6** as a brown solid (0.14 g, 48%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36 (4H, d, J=8.4 Hz), 6.98 (1H, s), 6.97 (1H, s), 6.62 (4H, d, J=8.4 Hz), 4.07 (2H, t, J=5.7 Hz), 3.88 (3H, s), 3.51 (2H, t, J=6.3 Hz), 2.10-1.92 (4H, m); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.0, 147.9, 146.5, 132.9, 119.3, 119.0, 115.3, 114.8, 114.1, 113.0, 92.9, 92.8, 86.6, 68.0, 56.0, 33.4, 29.4, 27.8; ESI-MS: [M+H$^+$] calcd for $C_{27}H_{26}BrN_2O_2$: 489.1172; found: 489.1173.

Compound 7: **Compound 6** (72 mg, 0.15 mmol), dimethylamine (5 mL, 30% aq.), acetone (5 mL) and ethanol (5 mL) were heated at 40 °C for 8 h. After cooled to room temperature, 100 mL of water was added to precipitate the crude product which was purified by recrystallization with DCM and PE to obtain **Compound 7** as a brown solid (53 mg 74%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (4H, d, J=8.1 Hz), 6.98 (1H, s), 6.97 (1H, s), 6.60 (4H, d, J=7.8 Hz), 4.05 (2H, t, J=6.6 Hz), 3.86 (3H, s), 3.83 (4H, br), 2.33 (2H, t, J=7.2 Hz), 2.23 (6H, s), 1.87 (2H, m), 1.64 (2H, m); ^{1}H NMR (400 MHz, acetone-d_6): δ 7.27 (4H, d, J=8.0 Hz), 7.02 (1H, s), 7.01 (1H, s), 6.67 (4H, d, J=7.8 Hz), 5.03 (4H, br), 4.07 (2H, t, J=6.0 Hz), 3.87 (3H, s), 2.30 (2H, t, J=6.4 Hz), 2.16 (6H, s), 1.84 (2H, m), 1.64 (2H, m); ^{13}C NMR (100 MHz, acetone-d_6): δ 149.9, 149.5, 133.4, 116.1, 115.1, 114.9, 111.7, 94.0, 87.0, 69.5, 59.9, 56.3, 45.7, 27.7, 24.8; ESI-MS: [M+H$^+$] calcd for $C_{29}H_{32}N_3O_2$: 454.2489; found: 454.2491.

Probe 1. A solution of **Compound 7** (53 mg, 0.12 mmol) and maleic anhydride (29 mg, 0.30 mmol) in 5 mL THF was stirred for 12 h. Afterwards acetic anhydride (5 mL) and sodium acetate (21 mg, 0.25 mmol) were added and the solution was heated at 80 °C for 0.5 h. After cooled to room temperature, the mixture directly went through a short column with ethyl acetate to remove acetic anhydride and the acetone to wash out the intermediate product.

After the solvent was removed, the intermediate product and bromoethane (3 mL) were dissolved in 10 mL of acetone and heated at 75 °C in a sealed tube for 8 h. The system was cooled to room temperature and 20 mL of PE was added to precipitate **Probe 1** as a yellow solid (55 mg, 65%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.66 (4H, d, J=8.0 Hz), 7.44 (4H, d, J=8.4 Hz), 7.25 (2H, s), 7.21 (4H, s), 4.14 (2H, t, J=5.6 Hz), 3.88 (3H, s), 3.37 (4H, m), 3.01 (6H, s), 1.83 (4H, m), 1.25 (3H, t, J=6.8 Hz,); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.6, 149.5, 148.4, 134.8, 131.7, 131.5, 126.7, 121.4, 117.7, 117.4, 115.4, 114.5, 91.4, 91.3, 89.0, 67.8, 61.9, 58.6, 55.9, 49.4, 25.4, 18.8, 7.7; ESI-MS: [M$^+$] calcd for $C_{39}H_{36}N_3O_6$: 642.2599; found: 642.2583.

Compound 9: A mixture of **Compound 8** (1.48 g, 2.9 mmol), I_2 (0.74 g, 2.9 mmol), KIO_3 (0.31 g, 1.45 mmol), acetic acid (50 mL),