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

Fan Gao, Hao Chen, Shu Xu, Yi Cheng, Yuguo Ma *

Beijing National Laboratory for Molecular Sciences (BNLMS), Key Lab of Polymer Chemistry & Physics of Ministry of Education, College of Chemistry, Peking University, Beijing 100871, China

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.

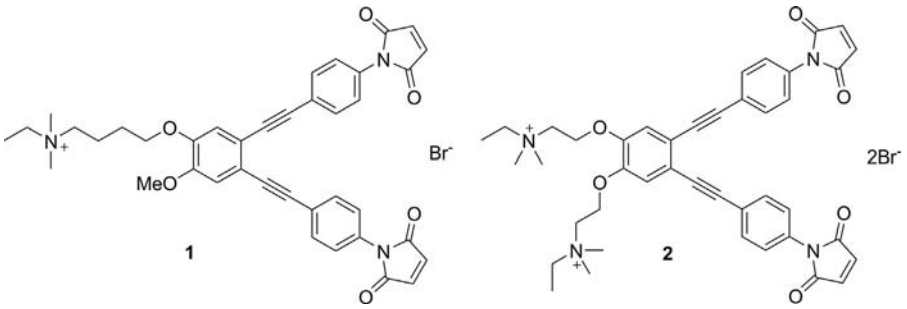
2. Experimental

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

* Corresponding author. Tel.: +86 10 6275 6660; fax: +86 10 6275 1708.
E-mail address: ygma@pku.edu.cn (Y. Ma).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
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**.

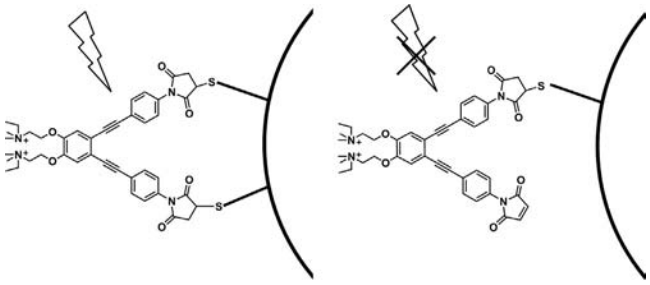


Fig. 1. A schematic illustration of how **Probe 2** responds to analyte molecules with a single (right) or two close (left) mercapto groups.

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.

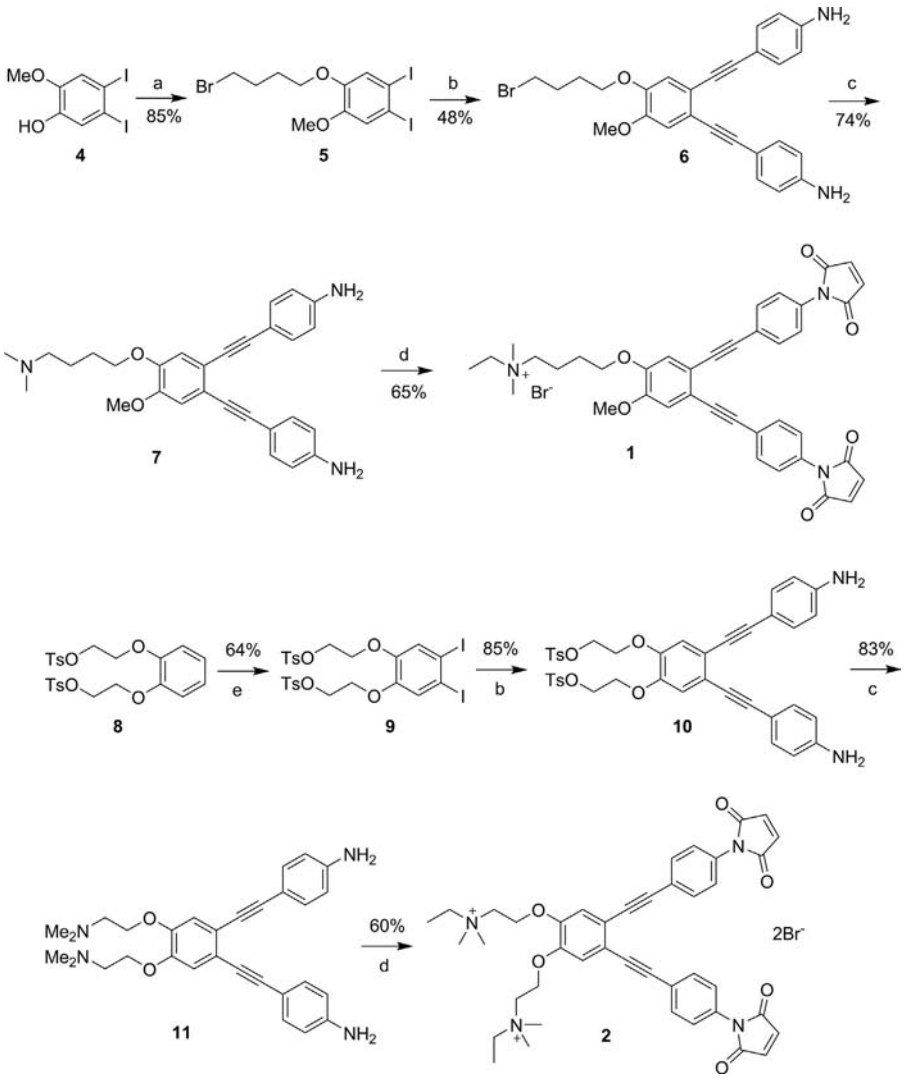
Compound 9: A mixture of **Compound 8** (1.48 g, 2.9 mmol), I_2 (0.74 g, 2.9 mmol), KIO_3 (0.31 g, 1.45 mmol), acetic acid (50 mL),

chloroform (20 mL), water (10 mL) and concentrated H_2SO_4 (5 mL) was stirred at 40 °C for 48 h. After being diluted with DCM (100 mL) and water (100 mL), the organic layer was washed with water, $NaHSO_4$ (aq.) and $NaHCO_3$ (aq.) sequentially. After being dried over $MgSO_4$, the solvent was removed and the crude product was purified by recrystallization from methanol to obtain **Compound 9** as a white solid (1.4 g, 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (4H, d, J=8.4 Hz), 7.34 (4H, d, J=8.4 Hz), 7.17 (2H, s), 4.32 (4H, t, J=4.8 Hz), 4.12 (4H, t, J=4.8 Hz), 2.45 (6H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.8, 145.1, 132.7, 130.0, 127.9, 125.9, 97.9, 67.9, 67.5 21.7; anal. calcd for $C_{24}H_{24}I_2O_8S_2$: C, 38.01; H, 3.19; found: C, 38.11; H, 3.27. EI-MS calcd for $C_{24}H_{24}I_2O_8S_2$: 757.9; found: 758.

Compound 10: Under N_2 protection, **Compound 9** (0.34 g, 0.45 mmol), 4-ethynylaniline (0.115 g, 0.98 mmol), $Pd(PPh_3)_2Cl_2$ (13 mg, 0.019 mmol) and CuI (5 mg, 0.026 mmol) were dissolved in 10 mL of THF and 3 mL of triethylamine and heated at 40 °C for 8 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 10** as a brown solid (0.28 g, 85%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.80 (4H, d, J=8.4 Hz), 7.37–7.31 (8H, m), 6.85 (2H, s), 6.63 (4H, d, J=8.4 Hz), 4.35 (4H, t, J=4.8 Hz), 4.17 (4H, t, J=4.8 Hz), 3.83 (4H, s), 2.43 (6H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 147.6, 146.8, 145.1, 132.9, 132.8, 130.0, 127.9, 120.5, 117.8, 114.8, 112.7, 93.5, 86.0, 67.9, 67.1, 21.7; ESI-MS: [M+H$^+$] calcd for $C_{40}H_{37}N_2O_8S_2$: 737.1986; found: 737.1987.

Compound 11: **Compound 10** (185 mg, 0.25 mmol), K_2CO_3 (50 mg.36 mmol), dimethylamine (5 mL, 30% aq.), acetone (5 mL) and ethanol (5 mL) were heated at 50 °C for 8 h. After cooled to room temperature, 100 mL of water was added to precipitate the crude product which was purified by re-crystallization with DCM and PE to obtain **Compound 11** as a brown solid (100 mg, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (4H, d, J=7.6 Hz), 6.98 (2 H, s), 6.62 (4H, d, J=7.6 Hz), 4.10 (4H, t, J=4.4 Hz), 3.81 (4H, br), 2.78 (4H, t, J=4.4 Hz), 2.34 (12H, s); ^{1}H NMR (400 MHz, acetone-d_6): δ 7.27 (4H, d, J=7.6 Hz), 7.04 (2H, s), 6.67 (4H, d, J=7.6 Hz), 5.03 (4H, br), 4.14 (4H, t, J=5.2 Hz), 2.70 (4H, t, J=5.2 Hz), 2.29 (12H, s); ^{13}C NMR (100 MHz, acetone-d_6): δ 149.8, 149.6, 133.4, 120.1, 116.7, 114.9, 111.7, 94.0, 87.0, 68.6, 58.9, 46.3; ESI-MS: [M+H$^+$] calcd for $C_{30}H_{35}N_4O_2$: 483.2754; found: 483.2749.

Probe 2. A solution of **Compound 11** (56 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



Scheme 2. Syntheses of **Probe 1 and Probe 2**.

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 2** as a yellow solid (60 mg, 60%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.66 (4H, d, J=8.4 Hz), 7.46 (4H, d, J=8.8 Hz), 7.41 (2H, s), 7.23 (4H, s), 4.58 (4H, t, J=4.4 Hz), 3.78 (4H, t, J=4.0 Hz), 3.50 (4H, q, J=7.2 Hz), 3.14 (12H, s), 1.31 (6H, t, J=7.2 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.6, 147.4, 134.8, 131.9, 131.6, 126.8, 121.2, 118.2, 116.3, 91.8, 88.7, 62.4, 61.2, 59.7, 50.3, 8.0; ESI-MS: [M^{2+}] calcd for $C_{42}H_{44}N_4O_6$/2: 350.1625; found: 350.1619.

Probe 3. N-(1-pyrenyl)maleimide (Probe 3) was synthesized according to literature [42]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.30–8.00 (7H, m), 7.84 (1H, d, J=8.0 Hz), 7.75 (1H, d, J=9.6 Hz), 7.04 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.2, 134.6, 132.1, 131.0, 130.7, 129.0, 128.6, 128.4, 127.1, 126.4, 126.3, 126.1, 125.9, 125.4, 125.1, 124.4, 121.4; ESI-MS: [M^+] calcd for $C_{20}H_{12}NO_2$: 298.0863; found: 298.0858.

2.3. Fluorescence spectroscopy

The excitation wavelength is 340 nm and the spectra were recorded over 350–600 nm. Probes, peptides and proteins were prepared as aqueous solutions with the concentration of 2×10^{-4} mol/L before fluorescence tests.

3. Results and discussion

3.1. Syntheses and structure characterization

The synthetic routes of **Probe 1 and Probe 2** are shown in Scheme 2. Maleimide is prone to hydrolysis under alkaline condition so quaternary ammonium salts cannot be introduced by reacting with trimethylamine after the maleimides are formed. On the other hand, the existence of quaternary ammonium salts complicates the purification process due to solubility in water and large polarity. It is not ideal to introduce the quaternary ammonium salts in the early stage. Therefore, an unconventional route is designed for the syntheses of these two molecules. We introduce a tertiary amine first, then the maleimides, and then transfer the tertiary amine to quaternary ammonium salts by reacting with bromoethane. The structures of both compounds were confirmed by ^{1}H NMR, ^{13}C NMR and high resolution ESI-MS.

Fig. 2 shows the ^{1}H NMR of **Probe 1 and Probe 2** in D_2O. Even though **Probe 1** is soluble in water with the solubility of about

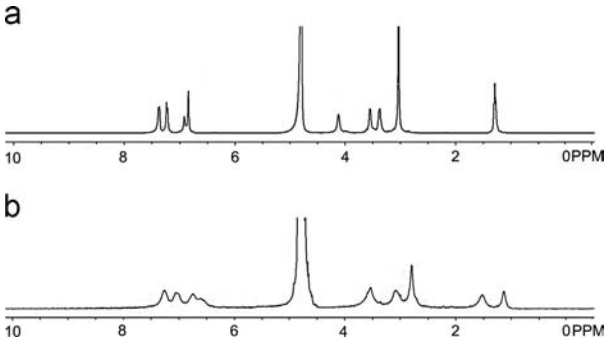


Fig. 2. (a) The ^{1}H NMR of **Probe 2** in D_2O. (b) The ^{1}H NMR of **Probe 1** in D_2O.

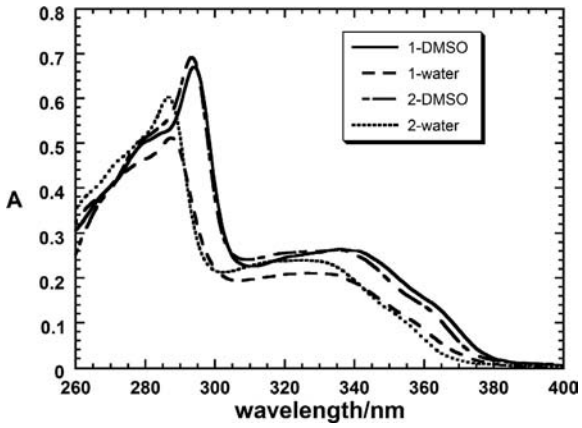


Fig. 3. UV spectra of **Probe 1** and **Probe 2** in DMSO and water at the concentration of 1×10^{-5} mol/L.

0.5 mg/mL, because of its large aromatic core, it aggregates strongly in aqueous solution indicated by the broadening of the NMR signals. By introducing one more ammonium ion, **Probe 2** has a better solubility of about 5 mg/mL and the aggregation is weaker as the NMR signals are much sharper than **Probe 1**'s. UV spectra (Fig. 3) also suggest that at the concentration of 1×10^{-5} mol/L, both **Probe 1 and Probe 2** aggregate and **Probe 1** has a stronger tendency of aggregation than **Probe 2**.

3.2. Peptide sensing

Probe 1 and Probe 2 are first tested in peptide sensing. Two peptides, **P1** and **P2**, with the sequences of LNSSPGCSME and LNCSPGCSME, respectively are designed and synthesized. The fluorescence of **Probe 1 and Probe 2** are quenched by the two terminal maleimide groups. The fluorescence recovers only when each maleimide group has reacted with a mercapto group. It is expected that **P1** with only one mercapto group will not respond to the probes while **P2** with two close mercapto groups will. Aqueous solutions of **P1** (2×10^{-6} mol/L) and **P2** (1×10^{-6} mol/L), with the same mercapto group concentration (2×10^{-6} mol/L), are prepared as the tested samples. A solution of Poly-L-Lysine (1×10^{-6} mol/L) serves as a blank control. Upon addition of **Probe 1** (1×10^{-6} mol/L), we observe significant fluorescence growing in both **P1** and **P2** solutions but no fluorescence in the blank control (Fig. 4a). Fluorescence in **P2** grows faster than **P1** at first. After 5 min, **P1** has a 2.7 times fluorescence growth and **P2** has an 8.5 times growth, 3.1 times stronger than **P1**. However, as fluorescence continues to grow in both samples, **P2**'s fluorescence reaches a plateau at 10–20 min and **P1**'s fluorescence eventually is as strong as **P2**'s (see Figure S1 in SI). The unexpected fluorescence recovery of sample **P1** can be explained by reaction of two peptide molecules to one probe molecule's two maleimide groups because a peptide is not bulky enough to prevent the reaction of the second peptide. Furthermore, reacting with a peptide alleviates the aggregation and promotes the reaction with the second peptide. **Probe 2** has better solubility. As a result, it not only has a stronger fluorescence response, but also has a better discrimination between **P1** and **P2** (Fig. 4b). After 5 min, the fluorescence grows 4.6 times and 31 times for **P1** and **P2**, respectively. The fluorescence intensity difference between **P2** and **P1** is 6.7 times for **Probe 2**. After the fluorescence test, we use ESI-MS to confirm the existence of the reaction products and find the corresponding molecular ion peaks ((**Probe 1**+2**P1**)$^{2+}$, (**Probe 1**+**P2**)$^{2+}$, (**Probe 2**+2**P1**)$^{3+}$, (**Probe 2**+**P2**)$^{3+}$) (see Figures S2 and S3 in SI).

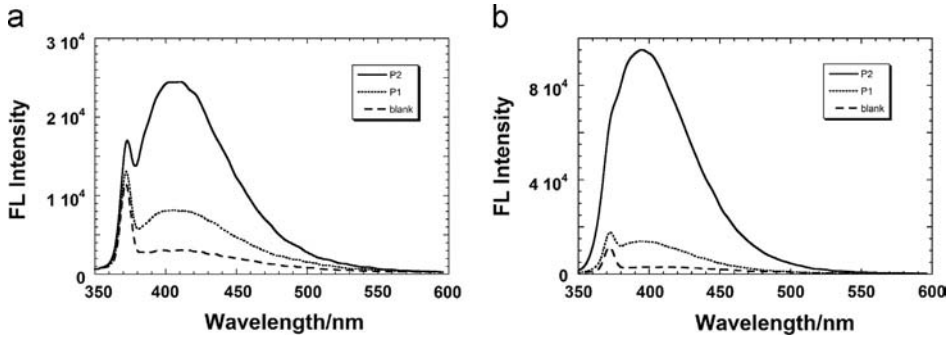


Fig. 4. Fluorescence response of **Probe 1** and **Probe 2** to peptides **P1** (2×10^{-6} mol/L, dot line), **P2** (1×10^{-6} mol/L solid line) and Poly-L-Lysine (1×10^{-6} mol/L, dash line), after mixing for 5 min. (a) Fluorescence response of **Probe 1** to the three peptides. (b) Fluorescence response of **Probe 2** to the three peptides. Concentration of **Probe 1** and **Probe 2** are 1×10^{-6} mol/L in aqueous solution.

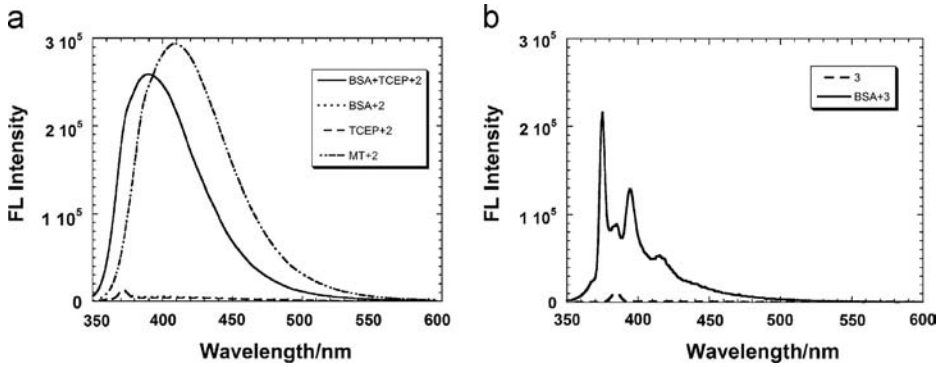


Fig. 5. (a) Fluorescence response of **Probe 2** to BSA (1×10^{-6} mol/L), BSA–TECP(1×10^{-6} mol/L, 1×10^{-6} mol/L) and MT (1×10^{-6} mol/L). (b) Fluorescence response of Probe 3 to BSA.

3.3. Protein detection

Since **Probe 2** has better sensing results than **Probe 1** due to its better solubility, we used **Probe 2** for the protein detection. We also used a reported mercapto probe, N-(1-pyrenyl)maleimide (**Probe 3**) as a Ref. [42]. **Probe 3** displayed intense fluorescence response toward mercaptan containing proteins like BSA, myosin, aldolase and thiolated galatin with the protein concentration of 2 mg/mL (ca. 10^{-5} mol/L). Bovine serum albumin (BSA) is a protein with one free mercapto group and 17 disulfide bonds. By mixing BSA with **Probe 3** we observed a strong fluorescence within 5 min indicating the existence of the free mercapto group (Fig. 5b). However, when mixing BSA with **Probe 2**, only a very slight fluorescence enhancement was observed, which is different from the result of peptide sensing (Fig. 5a). Because BSA has only one free mercapto group, only one maleimide of **Probe 2** reacts with the mercapto group, while the other one was not, due to the steric hindrance of the large protein, which still quenches the fluorescence. Therefore, one equivalent of tris(2-carboxyethyl)phosphine (TCEP) is added into the BSA solution to quantitatively break the disulfide bonds [22] and generate some free mercapto groups close to each other on one BSA molecules. Then **Probe 2** is added, within 5 min, a strong fluorescence emerged, due to both maleimide group are reacted (Fig. 5a). Figure S4 shows the fluorescence response to proteins solutions with different concentrations and significant fluorescence enhancement was observed at the concentration as low as 1×10^{-7} mol/L. These experiments proved that **Probe 2** can distinguish proteins with two close mercapto groups from those with a single mercapto group. Metallothioneins (MT) are a family of low molecular weight, cysteine-rich proteins with metal-binding and redox capabilities. They consist of about 60 amino acids including approximately 20 cysteine residues [43–45], so we consider **Probe 2** to be a suitable probe for MT. As shown in Fig. 5a, upon mixing with **Probe 2**, within 10 min, a strong fluorescence emerged, indicating the existence of MT. Figure S5 shows that **Probe 2** works under a wide range of pH, from 4 to 8.

4. Conclusion

In conclusion, we synthesize two water soluble probes, **Probe 1 and Probe 2**, with quaternary ammonium salts as solubilization groups. The fluorescent V-shape oligo(o-phenylene-ethynylene) backbone connects two maleimide groups as mercapto group recognition sites and fluorescence switches. The unique structure enables the probes to respond only to proteins with two close mercapto groups, but not to those with a single mercapto group. This work also introduces the concept of functional group combination detection. Optimization of emission wavelength and fluorescence quantum yield will further facilitate the application of these probes.

Acknowledgment

We are grateful for the financial support from the Ministry of Science and Technology (Basic Research Program 2013CB933501), the National Natural Science Foundation (No. 91227202, 21074004), and the Ministry of Education (NCET-08-0005) of China.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.07.032.

References

[1] B. Valeur, I. Leray, Chem. Rev. 205 (2000) 3–40.
[2] Z. Xu, J. Yoon, D.R. Spring, Chem. Soc. Rev. 39 (2010) 1996–2006.
[3] J. Wu, W. Liu, J. Ge, H. Zhang, P. Wang, Chem. Soc. Rev. 40 (2011) 3483–3495.
[4] J. Yang, T.M. Swager, J. Am. Chem. Soc. 120 (1998) 5321–5322.
[5] T. Andrew, T.M. Swager, J. Am. Chem. Soc. 129 (2007) 7254–7255.
[6] K. Shiraishi, T. Sanji, M. Tanaka, Mater. Interfaces 1 (2009) 1379–1382.
[7] X. Zhang, X. Qiu, R. Lu, H. Zhou, P. Xue, X. Liu, Talanta 82 (2010) 1943–1949.
[8] E.M. Nolan, S.J. Lippard, Chem. Rev. 108 (2008) 3443–3480.
[9] J.F. Zhang, Y. Zhou, J. Yoon, J.S. Kim, Chem. Soc. Rev. 40 (2011) 3416–3429.
[10] R. Métivier, I. Leray, B. Valeur, Chem. Commun. (2003) 996–997.
[11] M. Yu, M. Shi, Z. Chen, F. Li, X. Li, Y. Gao, J. Xu, H. Yang, Z. Zhou, T. Yi, C. Huang, Chem. Eur. J. 14 (2008) 6892–6900.
[12] Y. Xiang, Z. Li, X. Chen, A. Tong, Talanta 74 (2008) 1148–1153.
[13] H.-Y. Luo, J.-H. Jiang, X.-B. Zhang, C.-Y. Li, G.-L. Shen, R.-Q. Yu, Talanta 72 (2007) 575–581.
[14] Z. Xu, X. Chen, H.N. Kim, J. Yoon, Chem. Soc. Rev. 39 (2010) 127–137.
[15] S. Toal, K.A. Jones, D. Magde, W.C. Trogler, J. Am. Chem. Soc. 127 (2005) 11661–11665.
[16] H. Song, S. Rajendiran, N. Kim, S.K. Jeong, E. Koo, G. Park, T.D. Thangadurai, S. Yoon, Tetrahedron Lett. 53 (2012) 4913–4916.
[17] Y. Sun, Y. Liu, M. Chen, W. Guo, Talanta 80 (2009) 996–1000.
[18] X. Chen, Y. Zhou, X. Peng, J. Yoon, Chem. Soc. Rev. 39 (2010) 2120–2135.
[19] S. Sueda, S. Tanaka, S. Inoue, H. Komatsu, Anal. Biochem. 422 (2012) 52–54.
[20] E. Lu, X. Peng, F. Song, J. Fan, Bio. Med. Chem. Lett. 15 (2005) 255–257.
[21] M. Algarra, B.B. Campos, D. Gomes, B. Alonso, C.M. Casado, M.M. Arrebola, M.J. Diez de los Rios, M.E. Herrera-Gutiérrez, G. Seller-Pérez, J.C.G. Esteves da Silva, Talanta 99 (2012) 574–579.
[22] Q. Lin, C. Bao, A. Cheng, Y. Yang, W. Ji, L. Zhu, J. Am. Chem. Soc. 134 (2012) 5052–5055.
[23] B.K. McMahon, T. Gunnlaugsson, J. Am. Chem. Soc. 134 (2012) 10725–10728.
[24] X.-F. Guo, J.-B. Chen, H. Wang, H.-S. Zhang, W.-H. Huang, J. Guo, Talanta 99 (2012) 1046–1050.
[25] Z. Li, Z. Xue, Z. Wu, J. Han, S. Han, Org. Biomol. Chem. 9 (2011) 7652–7654.
[26] M. Bielecki, H. Eggert, J.C. Norrild, J. Chem. Soc. Perkin Trans. 2 (1999) 449–455.
[27] M. Ikeda, K. Fukuda, T. Tanida, T. Yoshii, I. Hamachi, Chem. Commun. 48 (2012) 2716–2718.
[28] A. Meister, Methods Enzymol. 251 (1995) 3.
[29] T. Finkel, Curr. Opin. Cell Biol. 15 (2003) 247–254.
[30] D.M. Townsend, K.D. Tew, H. Tapiero, Biomed. Pharmacother. 57 (2003) 145–155.
[31] L.A. Herzenberg, S.C. De Rosa, J.G. Dubs, M. Roederer, M.T. Anderson, S.W. Ela, S.C. Deresinski, L.A. Herzenberg, Proc. Natl. Acad. Sci. U. S. A 94 (1997) 1967–1972.
[32] A. Holmgren, Annu. Rev. Biochem. 54 (1985) 237–271.
[33] W. Qiu, L. Wang, L.W. Lu, A. Boechler, D.A.R. Sanders, D. Zhong, Proc. Natl. Acad. Sci. U. S. A 104 (2007) 5366–5371.
[34] J. Drenth, J.N. Jansonius, R. Koekoek, H.M. Swen, B.G. Wolthers, Nature 218 (1968) 929–932.
[35] B.A. Griffin, S.R. Adams, R.Y. Tsien, Science 281 (1998) 269–272.
[36] R.A. Scheck, A. Schepartz, Acc. Chem. Res. 44 (2011) 654–665.
[37] T.L. Halo, J. Appelbaum, E.M. Hobert, D.M. Balkin, A. Schepartz, J. Am. Chem. Soc. 131 (2009) 438–439.
[38] F. Gao, E. Yao, W. Wang, H. Chen, Y. Ma, Phys. Chem. Chem. Phys. 14 (2012) 15321–15327.
[39] Z. Peng, Y. Pan, B. Xu, J. Zhang, J. Am. Chem. Soc. 112 (2000) 6619–6623.
[40] G. Topal, N. Demirel, M. Toğrul, Y. Turgut, H. Hoşgören, J. Heterocycl. Chem. 38 (2001) 281–284.
[41] P.R. Serwinski, P.M. Lahti, Org. Lett. 5 (2003) 2099–2102.
[42] J.K. Weltman, R.P. Szaro, A.R.J. Frackelton, R.M. Dowben, J.R. Bunting, R.E. Cathou, J. Biol. Chem. 248 (1973) 3173–3177.
[43] P. Coyle, J.C. Philcox, L.C. Carey, A.M. Rofe, Cell. Mol. Life Sci. 59 (2002) 627–647.
[44] D.H. Hamer, Ann. Rev. Biochem. 55 (1986) 913–951.
[45] P. Babula, M. Masarik, V. Adam, T. Eckschlager, M. Stiborova, L. Trnkova, H. Skutkova, I. Provaznik, J. Hubalek, R. Kizek, Metallomics 4 (2012) 739–750.