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# Thiol-Reactive Molecule with Dual-Emission-Enhancement Property for Specific Prestaining of Cysteine Containing Proteins in SDS-PAGE

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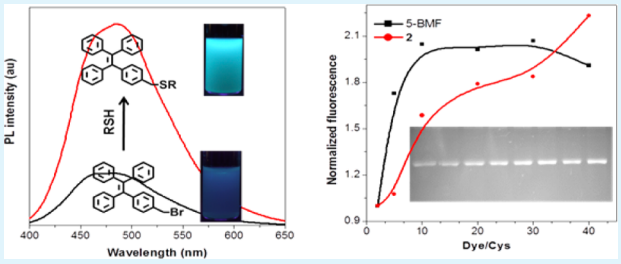
Supporting Information

**ABSTRACT:** 1-[4-(Bromomethyl)phenyl]-1,2,2-triphenylethene (**2**) was synthesized and evaluated for specific fluorescent prestaining of proteins containing cysteine (Cys) in SDS-PAGE. The molecule showed classic aggregation-induced emission (AIE) property in protein labeling and its quantum efficiency was further enhanced upon reacting with Cys. The parameters of reaction such as labeling time and concentration of dye and reducing reagent-tris(2-carboxyethyl)phosphine (TCEP) were examined to obtain the optimal labeling condition. In addition to its specific labeling effect, molecule **2** also showed its advantage over traditional self-quenching dyes through labeling Cys containing BSA with different dye/Cys ratios.




**KEYWORDS:** *tetraphenylethylene, fluorescent enhancement, Cys staining, thiol-reactive, aggregation-induced emission*

## INTRODUCTION

Fluorescent labeling of proteins has been identified as one of the most attractive methods for biological detections such as protein tracing in biological processes and quantification of proteins after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[1−3] Protein samples in SDS-PAGE gel could be labeled covalently or noncovalently by the fluorescent dyes. Poststaining employs both covalent and noncovalent dyes to label the protein samples after electrophoresis and has an excellent limit of detection (LOD) in most cases. However, poststaining method requires a time-consuming gel staining and destaining process before getting the desired fluorescent bands of the proteins in the gel. Compared with poststaining, covalent labeling of protein samples before electrophoresis has a much shorter staining process, and a better signal-to-noise ratio in its application as prestaining method for protein analysis in gel.[4,5]

By utilizing chemical reactions between the reactive groups of the fluorescent dyes and specific amino acids of proteins, two main methods have been developed for prestaining of proteins for SDS-PAGE. Amine-reactive fluorescent dyes, such as fluorescein isothiocyanate (FITC), Cy3 and Cy5, have been applied to label the lysine (Lys) residues of protein samples and used successfully in protein detection on gel.[6,7] On the other hand, thiol-reactive fluorescent compounds with alkyl halide groups, such as 5-bromomethylfluorescein (5-BMF), are utilized to label the cysteine (Cys) residues of thiol-containing proteins.[8]

Because most proteins have more Lys than Cys, proteins labeled by amine-reactive dyes usually possess higher fluorescence than those labeled by thiol-reactive dyes. However, overlabeling of the abundance of Lys residues could decrease the water solubility of the labeled proteins and significantly change the mobility of proteins in gel during electrophoresis.[9]

In comparison with Lys labeling, prestaining of Cys residues offers a number of advantages. The occurrence of Cys residues in a protein is much less frequent than other amino acids so that there is little chance of overlabeling. By saturating the reactive sites, Cys labeling can lead to more reproducible quantitative labeling and possesses a lower impact on protein solubility. Cys is highly reactive toward the dyes[10] and the resulting alkylated Cys shows only marginal effect on the mobility of proteins in gel. Moreover, Cys residues are not the cleaving site of commonly available proteases and hence the protein modification has little effect on the peptide maps for future analysis by mass spectrometry after SDS-PAGE.

A significant amount of effort has been put into the development of luminescent materials with aggregation-induced emission (AIE) property for cell labeling, DNA

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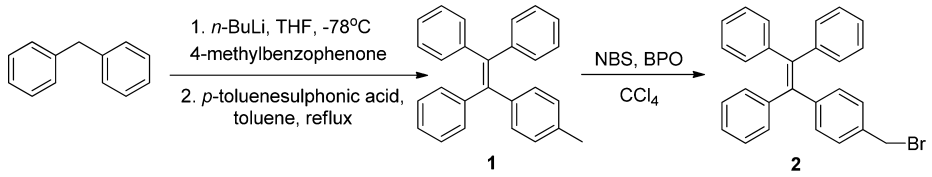
**Scheme 1. Synthesis of 1-[4-(Bromomethyl)phenyl]-1,2,2-triphenylethene (2)**




detection, protein detection with an amine-reactive tetraphenylethylene (TPE) derivative.[11−24] In this work, a thiol-reactive tetraphenylethylene (TPE) derivative **2** has been prepared and applied for the specific prestaining of proteins with Cys residues in SDS-PAGE gel. In addition to its AIE property, **2** can be even more emissive after reaction with thiol-containing compounds in aqueous media. The specificity of labeling Cys residues with **2** and the optimal labeling conditions were thoroughly examined.

## RESULTS AND DISCUSSION

The thiol-reactive TPE derivative **2** was prepared according to the synthetic route shown in Scheme 1. Similar to all other AIE molecules, **2** was weakly emissive in good solvents and possessed a 50-fold higher fluorescence intensity when dissolved in the water/THF solution with a water fraction of 90 vol% (see Figure S1 in the Supporting Information). The quantum efficiency of **2** was 31.7% in the solid state and was 1.4% in the water/THF solution with a water fraction of 90 vol % (quinine sulfate in 0.1 M $H_2SO_4$ as reference).

In addition to the enhancement of fluorescence because of its AIE property, **2** becomes even more emissive through the reaction between its bromomethyl group and the thiol group of proteins, presumably due to the elimination of internal heavy atom effect of bromide.[25] Dithiothreitol (DTT) was selected as the model reactant to react with **2** to demonstrate this fluorescence enhancement effect. PL intensity of 10 $\mu$M of **2** was measured in a THF/water mixture with a water fraction of 90 vol% and then an equal amount of DTT was added. The mixture was then incubated at room temperature overnight and the PL intensity was measured again. As shown in Figure 1, the PL peak intensity of **2** in the water/THF solution with a water fraction of 90 vol% was approximately 3 times higher after reacting with thiol. The signal-to-noise ratio would benefit from this double-enhancement effect in prestaining of SDS-PAGE gel.

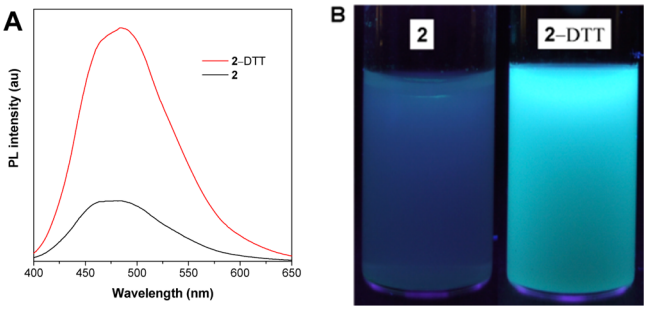



**Figure 1.** (A) PL spectra of **2** before and after the reaction with DTT. The suspension medium was a THF/water mixture with a water fraction of 90 vol%; concentration of **2**:10 $\mu$M; excitation wavelength: 320 nm. (B) Photo of **2** before and after the reaction with DTT in a THF/water mixture with a water fraction of 90% taken under 365 nm UV irradiation.

To verify the time-dependence of prestaining efficiency, we labeled bovine serum albumin (BSA) samples with **2** at 50 °C for different durations ranging from 10 min to 4 h and analyzed after SDS-PAGE.[26] The fluorescence intensity of the prestained BSA increased as the labeling time was extended. The maximum intensity under specific labeling conditions was observed after 3 h and remained unchanged even with an additional 1 h of reaction time (Figure 2A). On the basis of this

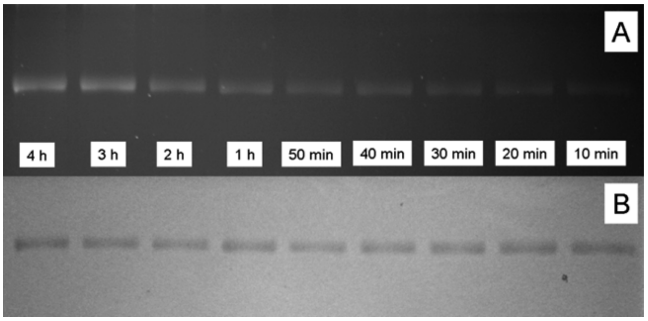



**Figure 2.** (A) SDS-PAGE fluorescence image of **2** labeled BSA with different labeling times and (B) image of the same gel restained with Coomassie R-250. Quantity of BSA per band: ~1.5 $\mu$g.

result, reaction time should be controlled within 4 h to avoid any undesired nonspecific labeling. The time-dependent labeling effect provides a way to determine the desirable degree of labeling (DOL) according to different applications by simply controlling the labeling time.

BSA samples were then subjected to labeling with different dye concentrations. The SDS-PAGE fluorescence intensity of labeled BSA increased with increasing dye concentration. The maximum intensity was reached at a dye concentration of 0.5 mM or above (see Figure S2A in the Supporting Information). Further increasing the dye concentration should be avoided because of nonspecific labeling. Clearly, the DOL of protein samples can be controlled by varying the dye concentration. It is notable that the migrating rates of the BSA labeled by **2** with different concentrations were comparable with that of the pure denatured BSA sample on the same SDS-PAGE gel (see Figure S2B in the Supporting Information). This indicates that labeling Cys residues of the protein with **2** had little effect on its mobility in gel.

The thiol groups of proteins usually exist as a disulfide bond that needs to be completely reduced to enable effective labeling using reducing reagents such as DTT, $\beta$-mercaptoethanol or tris(2-carboxyethyl)phosphine (TCEP). DTT and $\beta$-mercaptoethanol are reagents traditionally used for the reduction of disulfide bonds. However, they could react with the thiol-reactive fluorescent dyes and need to be removed prior to fluorescent labeling. Phosphines such as TCEP usually have a much lower reactivity toward thiol-reactive compounds and do not need to be removed prior to the alkylation reaction. Hence, in order to obtain the desired labeling effect, we set out to

explore the optimal concentration of TCEP in the labeling reaction. From the results shown in Figure S3A in the Supporting Information, among all the BSA samples reduced with various concentrations of TCEP, reduction of BSA with 1 mM TCEP produced the highest fluorescence intensity after labeling with **2**. Lower concentrations of TCEP only gave incomplete reduction of disulfide bonds; higher concentrations induced the undesired reaction of **2** and TCEP. As a control experiment, the sample without treating with TCEP prior to labeling showed no emission at all (see Figure S3A in the Supporting Information). After restaining the gel with coomassie brilliant blue (CBB), it was observed that this sample without treatment with any reducing reagent before SDS-PAGE separation showed a higher migration rate on gel because it still kept the native folding structure stabilized by disulfide bonds (see Figure S3B in the Supporting Information).

Urea can completely denature the proteins for effectively labeling and protein quantification. The concentration of urea commonly used for protein denaturation is 8 M. However, a lower concentration could eliminate desalting process before further applying the fluorescent labeling product. Protein samples were labeled with various concentrations of urea at 50 °C for 3 h with agitation. BSA samples were all labeled successfully with **2** when treated with varying concentrations of urea before labeling (Figure 3A). The difference of fluorescence intensities among the bands was due to the various sample quantities loaded on each lane (Figure 3B). The results indicated that BSA could be denatured completely without using any denaturants under the high temperature labeling conditions stated above.

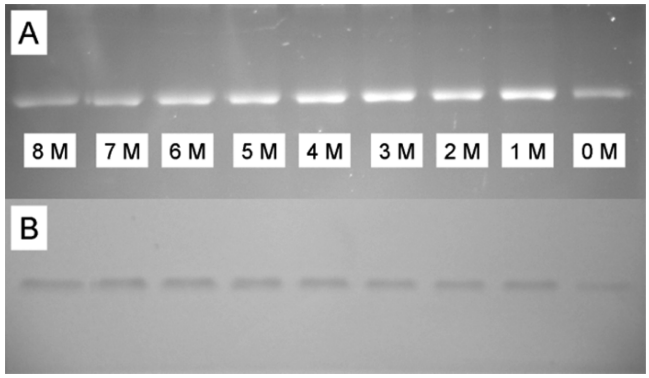



**Figure 3.** (A) SDS-PAGE fluorescence image of **2** labeled BSA in the presence of different concentrations of urea and (B) image of the same gel restained with Coomassie R-250. Quantity of BSA per band: ~1.5 μg.

Specific labeling of Cys residues by **2** was examined through the labeling of proteins with different Cys contents. BSA (35 Cys), yeast enolase (single Cys) and horse myoglobin (0 Cys) were labeled with 0.5 mM of **2**, respectively. The corresponding fluorescent products were analyzed on gel. BSA with 35 Cys residues displayed a much higher emission than yeast enolase, which has only one Cys residue. Horse myoglobin, a protein without any Cys residue in its structure, showed no fluorescence at all (Figure 4A). The results further confirmed the specificity of **2** to Cys residue when the working concentration of 0.5 mM was used. The results from CBB staining showed that the three proteins had similar loading on the gel (Figure 4B).

The comparison of protein labeling effect between self-quenching dyes and the novel AIE dye was demonstrated by using 5-BMF and **2**. BSA was labeled by each dye with a series of varying dye/Cys ratios. After the electrophoresis, the gel fluorescent image was taken under UV excitation and the fluorescence intensity was obtained from three independent experiments. Fluorescence intensities normalized with the sample with the lowest dye concentration are plotted versus the dye/Cys ratio (Figure 5). The fluorescence intensity of **2** labeled protein samples kept on increasing (Figure 5A), while self-quenching effect was observed when 30 times of 5-BMF was used to label the Cys residues (Figure 5B). This self-quenching effect is inevitable for self-quenching dyes such as 5-BMF and limits these dyes' application on quantification of proteins. In contrast, detection and quantification of proteins with high contents of Cys can be easily achieved by using AIE active molecules such as **2**.

## CONCLUSION

In conclusion, a new thiol-reactive tetraphenylethylene derivative (**2**) with AIE characteristics has been prepared and evaluated. The quantum yield of **2** in aqueous media could be further enhanced by reacting with thiol groups, which could improve the signal-to-noise ratio in the application of SDS-PAGE gel staining. Molecule **2** was capable of labeling Cys residues of proteins by using the optimal labeling time,

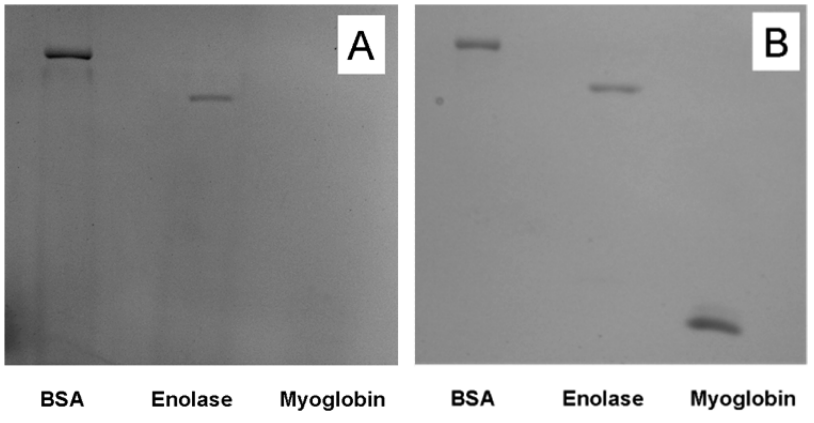



**Figure 4.** (A) Inverted SDS-PAGE fluorescence image of **2** labeled proteins with various contents of Cys residue and (B) image of the same gel restained with Coomassie R-250. Quantity of protein per band: ~1.5 μg.

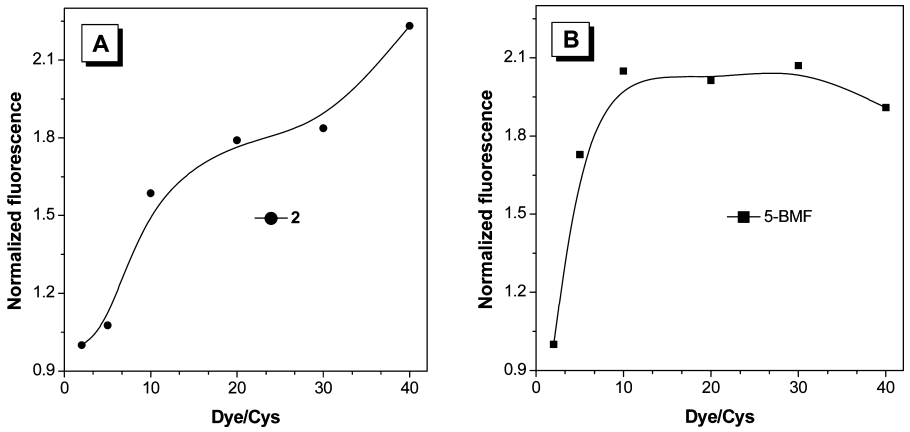



**Figure 5.** Plots of normalized fluorescence of labeled BSA versus dye/Cys ratio. BSA samples were by (A) **2** and (B) 5-BMF.

concentration of **2** and TCEP. By using a sufficiently high temperature, denaturants such as urea were not required. Three proteins with different contents of Cys were labeled by **2** and the fluorescence intensity decreased along with the decreasing Cys content. The observed fluorescence intensity/Cys content relationship confirmed the specific labeling effect of **2** to Cys residues. The AIE property of **2** showed no aggregation-caused quenching effect at a high dye/Cys ratio.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental details including synthesis, prestaining and SDS-PAGE protocol, AIE property of **2**, fluorescent images of BSA labeled with different concentrations of **2** and with different concentrations of TCEP. 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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