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An amine-reactive tetraphenylethylene derivative for protein detection in SDS-PAGE

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A new aggregation-induced emission (AIE) compound 1,2-bis[4-(isothiocyanatomethyl)phenyl]-1,2-diphenylethene (**2**) was synthesized for use in SDS-PAGE. The molecule is practically nonemissive in solution but becomes highly emissive after reacting with the amine groups of the proteins by either the prestaining or poststaining method. The sensitivity of **2** achieved in the prestaining method is the same as that of Coomassie brilliant blue (CBB), while that observed in the poststaining method is higher than that of CBB. Excellent linear responses with the amount of protein were obtained in both cases. The detection of a mixture of proteins with different molecular weights was successfully achieved.

Introduction

Polyacrylamide gel electrophoresis (PAGE) is a widely used method for the analysis of proteins due to its high resolution, capability to handle multiple samples simultaneously, ability to analyze a trace amount of protein and modest cost.[1–4] However, the quantification of an exceedingly low level of proteins in PAGE is still a major challenge.

Numerous commercial dyes are available for the detection of proteins in SDS-PAGE gels. Because of their optical visibility and ease of use,[5–8] colorimetric stains such as Coomassie brilliant blue (CBB) and silver stain are widely used to stain the proteins through poststaining after the electrophoresis step, which is adopted for all noncovalent and most covalent stains. However, CBB suffers from a lack of sensitivity, poor signal-to-noise ratio due to high staining background and needs a multistep staining procedure. Silver staining is known for its inferior reproducibility, high background noise and incompatibility with mass spectrometry due to the fixation step during staining.

Fluorescent dyes with high sensitivity and excellent signal-to-noise ratio have become increasingly attractive in protein staining in PAGE. Fluorescent dyes such as SYPRO Red binding noncovalently and unspecifically to the SDS (sodium dodecyl sulfate) surrounding the proteins in gel are popular in the poststaining of SDS-PAGE.[9–11] Compared with colorimetric stains, fluorescent dyes offer a higher sensitivity, a wider linear range and a shorter staining procedure.[12] However, similar to the colorimetric stains, the poststaining method employed by most traditional fluorescent dyes also requires the labeled proteins to be fixed with dilute acetic acid. This inhibits the transfer of the proteins on the gel to the nitrocellulose membranes for further analysis during Western blotting.

Alternatively, many fluorescent dyes are also available to covalently label the proteins prior to electrophoresis in the prestaining method. The gels can be analyzed immediately after electrophoresis without any further treatment. Amine-reactive fluorescein isothiocyanate (FITC) and thiol-reactive 5-bromomethylfluorescein (5-BMF) are commonly used to form stable conjugates with proteins by their functional groups and obtain fairly high sensitivity through the simple prestaining method.[13,14] The cyanine based dyes, such as CyDye derivatives, could covalently prestain various proteins with different CyDyes at the same time and enable the comparison of different samples in one gel run.[12] However, covalently prestaining has the risk of changing the migration rate of proteins in electrophoresis.

Apart from the disadvantages of both prestaining and poststaining methods mentioned above, the nature of conventionally used fluorescent dyes significantly embarrassed their application in protein staining. All the covalently binding aggregation-caused-quenching (ACQ) dyes face the self-quenching problem when the fluorophore to protein ratio (*F*/*P* ratio) is over a certain level.[15]

A great deal of effort has been made on the development of luminescent materials with the aggregation-induced emission (AIE) property.[16–18] In this work, an amine-reactive tetraphenylethylene (TPE) derivative **2** has been designed and applied for the detection of proteins in SDS-PAGE by both prestaining and poststaining. Its AIE property eliminates the *F*/*P* ratio restriction, allowing the use of relatively high concentrations of fluorophore in both covalent prestaining and poststaining methods. Furthermore, since both staining methods require no fixation procedure, the stained proteins can be further analyzed.

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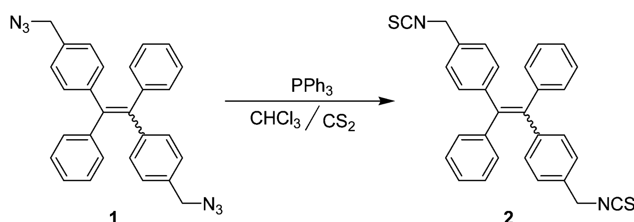
Results and discussions

The tetraphenylethylene derivative **2** was prepared according to the synthetic route shown in Scheme 1. Compared with the more reactive isocyanate compounds, isothiocyanate products are more stable in water and in most organic solvents, but have a comparable reactivity with the amine groups of proteins. This stability feature prompted us to synthesize tetraphenylethylene derivatives with isothiocyanate groups such as compound **2** for protein labeling and further bioassay. Different from the conventional fluorescent dyes which often suffer from the concentration-dependent self-quenching, **2** was expected to have no concentration limit due to its AIE property.

The AIE property of **2** was demonstrated in Fig. 1. Similar to all the other AIE molecules, **2** was weakly emissive in good solvents such as THF, while the addition of water, a non-solvent, dramatically increased its emission efficiency (Fig. 1A). With water content ranging from 0 to 70%, the fluorescence intensity remained at a very low level. When the water concentration was in the 80–90 vol% range, a rapid and dramatic increase of the fluorescence intensity with increasing water content was observed (Fig. 1B). The photoluminescence (PL) intensity of **2** in the water–THF solution with a water fraction of 90 vol% was approximately 50-fold higher than that in the mixture with 70% water. The quantum efficiency of **2** was 52.2% in the solid state.

Since nearly all kinds of proteins have reactive amine groups (lysine residues) on their molecular chains, bovine serum albumin (BSA), a commonly used protein, was employed as a test sample to verify the staining effect of compound **2**. The prestained BSA was loaded onto the gel lane a and the pure BSA sample was loaded onto lane b at the same time as a control. After electrophoresis, the gel fluorescent image was taken under UV excitation within the Gel Doc XR+ documentation system (Fig. 2A). As is expected, the prestained sample in lane a was highly emissive just after the electrophoresis due to the presence of **2** chemically bound with BSA while the pure BSA sample in lane b was nonemissive. The gel was then introduced to the poststaining procedure and the fluorescent image was again obtained (Fig. 2B). The emission from the band in lane b after poststaining demonstrated the success of the reaction between **2** and BSA on the gel. Both emissive bands on the gel were shown to be of BSA from CBB staining results (Fig. 2C). These results showed that compound **2** reacted with the amine group of the proteins very efficiently *via* both prestaining and poststaining methods. The position of the BSA bands in lanes a and b is the same, indicating that **2** has no significant effect on the protein migration rate.



Scheme 1 Synthesis of 1,2-bis[4-(isothiocyanatomethyl)phenyl]-1,2-diphenylethene (**2**).

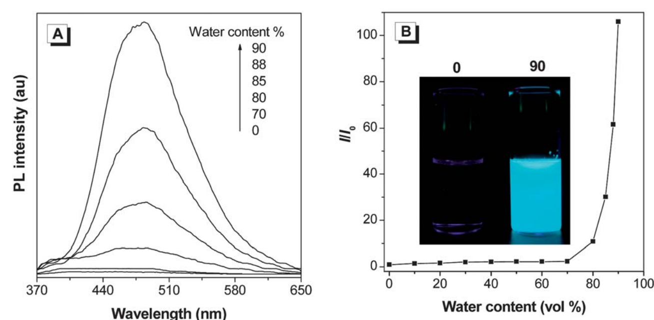


Fig. 1 (A) PL spectra of **2** in THF–water mixtures with different water contents; concentration of **2**: 10 μM; excitation wavelength: 320 nm. (B) Plot of PL peak intensity *versus* water content of the THF–water mixture. I_0 is the PL intensity in pure THF solution. Inset of (B): photo of THF–water mixtures of **2** with 0 and 90 water fractions taken under 365 nm UV irradiation.

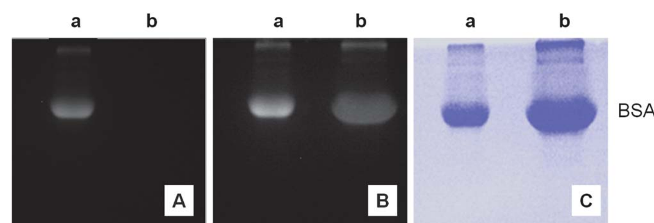


Fig. 2 (A) SDS-PAGE fluorescence image of **2** prestained BSA (lane a) and pure BSA (lane b), (B) fluorescence image of the same gel poststained with **2** for 3 h and (C) image of the same gel restained with Coomassie R-250.

A comparison between compound **2** poststaining and CBB staining was carried out. The entire poststaining and electrophoresis processes were repeated with a series of progressively diluted BSA samples. Under identical staining conditions, the emission of a band was directly proportional to the loaded amount of protein and thus decreased with the dilution of the protein samples (Fig. 3A). Compound **2** poststaining achieved a limit of detection (LOD) as low as 0.1 μg in this gel electrophoresis process while the LOD for CBB staining is 0.2 μg (Fig. 3B). This observation means compound **2** has a higher sensitivity than CBB *via* a similar poststaining process.

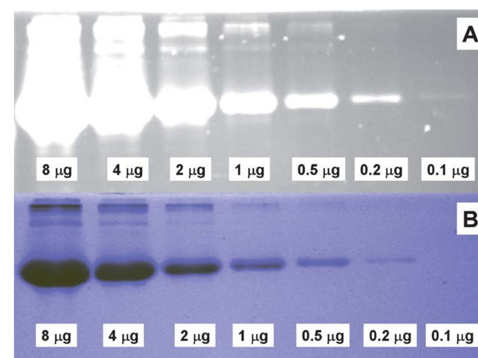


Fig. 3 (A) SDS-PAGE fluorescence image of **2** poststained BSA with various loaded amounts and (B) image of the same gel restained with Coomassie R-250.

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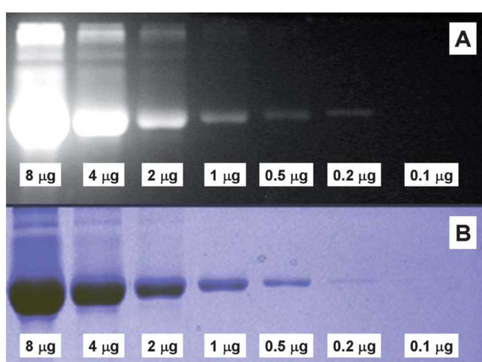

Fig. 4 (A) SDS-PAGE fluorescence image of **2** prestained BSA with various loaded amounts of protein and (B) image of the same gel restained with Coomassie R-250.

The prestaining sensitivity of compound **2** was evaluated. The sensitivity was lowered to 0.2 μg (Fig. 4A) *via* fluorescent prestaining due to the decrease in the amount of compound **2** bound with the BSA through a shorter labeling process. The CBB restaining of the same gel showed the same results as those from the compound **2** prestaining (Fig. 4B). However, CBB staining was time consuming and had a strong background with the gel image.

The linearity of response was investigated for both the prestaining and poststaining methods. Fluorescence intensities were obtained from a series of progressively diluted BSA samples with three independent experiments for each sample. The bands with the highest amount of BSA were used as the reference and a plot of the normalized fluorescence *versus* the amount of protein was obtained (Fig. 5). Excellent linear responses with good correlation coefficients were achieved from 0.5 to 8 μg for prestaining and 0.2 to 8 μg for poststaining. The results enabled the detection and quantification of proteins on the PAGE gel by comparing the fluorescence intensities between a sample and a standard in the same gel.

A comparison of the protein labeling effect on gel between traditional self-quenching dyes and the novel AIE dye was demonstrated using FITC and **2** with the prestaining method. An equal amount of BSA was labeled with a series of concentrations of FITC and of **2**. After the electrophoresis, the gel fluorescent image was taken under UV excitation. Fluorescence intensities were obtained from three independent experiments. Fluorescence intensities normalized with the sample with the lowest dye concentration are plotted *versus* the *F*/*P* ratio (Fig. 6). A self-

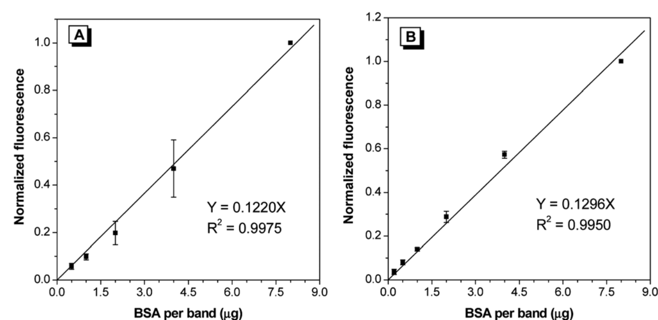

Fig. 5 Plots of normalized fluorescence *versus* amount of BSA in the SDS-PAGE assay. (A) Prestained by **2** and (B) poststained by **2**.

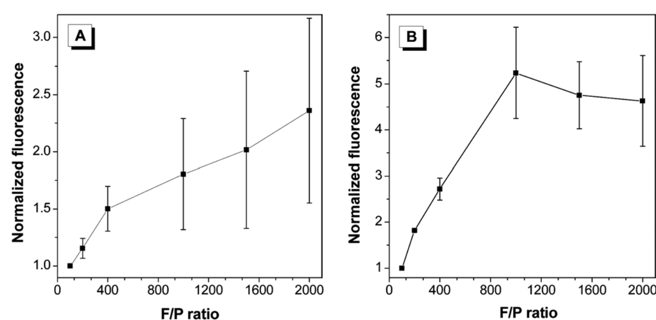

Fig. 6 Plots of normalized fluorescence *versus* *F*/*P* ratio in the SDS-PAGE assay *via* the prestaining method by (A) **2** and (B) FITC.

quenching effect was observed when 1000-fold of FITC was used to label protein samples, while the fluorescence intensities of the **2** labeled protein samples kept increasing. This self-quenching effect is inevitable for aggregation-caused quenching dyes such as FITC and hence limits the application of such dyes on quantification of proteins on gel.

To mimic the real SDS-PAGE staining process, three proteins with different molecular weights (BSA, 66 kDa, glucose 6-phosphate dehydrogenase, 57 kDa and chymotrypsinogen, 25 kDa) were mixed together and equally divided into two portions. One portion was prestained and loaded into lane a of the gel, while the other portion without any staining was loaded into lane b. The fluorescent image of the prestained sample was taken right after electrophoresis (Fig. 7A). Three fluorescent bands which represent the three different proteins were observed in lane a. The sample in lane b was not prestained and thus invisible. The poststaining method was then applied to the same gel, followed by fluorescence imaging. Fluorescent bands observed in lane b after poststaining were the same as the prestained bands in lane a (Fig. 7B). From these results, it was confirmed that different proteins could be labeled by both the prestaining and poststaining methods and all the emissive bands were located at the same position. The AIE dye with two reactive groups did not crosslink different proteins because the steric effect of protein molecules inhibited the crosslinking of proteins during prestaining. It can also be seen that the image from prestaining possessed a lower signal-to-noise ratio than that from poststaining.

Without fixing the proteins on gel, the transfer of labeled proteins from gel to nitrocellulose membranes can be achieved for both prestaining and poststaining methods. The fluorescent images of the membranes were taken immediately after transfer

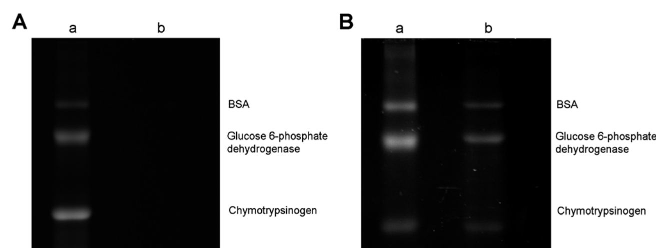

Fig. 7 (A) SDS-PAGE fluorescence image of **2** prestained sample with various proteins (lane a) and pure proteins (lane b), (B) fluorescence image of the same gel poststained with **2** for 3 h.

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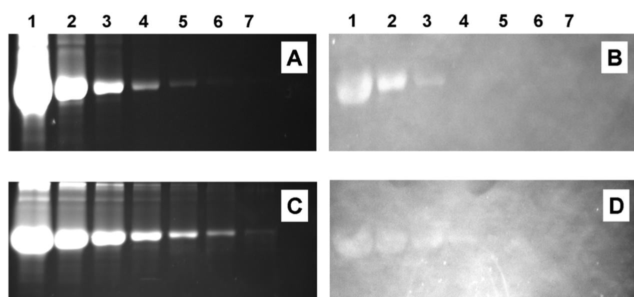

Fig. 8 Stained BSA samples were transferred onto the nitrocellulose membranes. (A) and (C) represent the SDS-PAGE fluorescence images of prestained and poststained BSA with various loaded amounts of protein; (B) and (D) represent the fluorescent images of the BSA samples on membranes. Lanes 1–7 represent the bands of the labeled BSA with the following loaded amounts: 8, 4, 2, 1, 0.5, 0.2 and 0.1 μg.

(Fig. 8B and D) and compared with the images of the gels (Fig. 8A and C). The semitransparent nitrocellulose membrane weakened the excitation UV light from its backside and therefore the sensitivities on the other side were much lower than that on the gels. The LODs on nitrocellulose membrane were 2 μg for prestaining (Fig. 8B) and 1 μg for poststaining (Fig. 8D). The success of protein transfer for both prestaining and poststaining methods offers the possibility of further investigation of labeled proteins.

Conclusion

In this work, we synthesized a new amine-reactive tetraphenylethylene derivative (**2**) with AIE characteristics and demonstrated its ability for the detection of a low quantity of proteins in the SDS-PAGE gel. For the prestaining method, the detection performance of compound **2** was comparable to CBB staining but it required a very short staining time. In poststaining, compound **2** offered a better limit of detection using the same protocol as that for CBB staining. Both prestaining and poststaining methods offered a wide linear detection range. In addition, unlike the traditional aggregation-induced quenching dyes, the AIE property of **2** did not suffer from any *F*/*P* ratio limit. There was no obvious crosslinking and the transfer to membrane worked well for both prestained and poststained samples. Non-fixation staining protocols enable the transfer of labeled proteins to nitrocellulose membranes for further analysis.

Experimental

General information

Acrylamide was purchased from Bio-Rad. Other chemicals, reagents and solvents were all purchased from Aldrich or Invitrogen. NMR spectra were taken on a Bruker ARX 400 NMR spectrometer using $CDCl_3$ as the solvent. HRMS spectra were recorded on a Finnigan TSQ 7000 triple quadrupole spectrometer operating in a MALDI-TOF mode. Photoluminescence (PL) spectra were recorded on a Perkin-Elmer LS 55 spectrofluorometer. The quantum efficiency of **2** was measured using an integrating sphere. Compound **1** was synthesized according to our previous work.[19]

Synthesis of 1,2-bis[4-(isothiocyanatomethyl)phenyl]-1,2-diphenylethene (2)

To a solution of 1,2-bis[4-(azidomethyl)phenyl]-1,2-diphenylethene **1** (110.5 mg, 0.25 mmol) and CS_2 (0.24 mL, 4.0 mmol) in $CHCl_3$ (1.0 mL) was added PPh_3 (131.1 mg, 0.5 mmol) at room temperature. The mixture was stirred for 1.5 h. After solvent evaporation under reduced pressure, the crude product was purified by a silica gel column using ethyl acetate–hexane (1 : 10 v/v) as the eluent. A white powder of **2** was obtained in 60% yield. ^1H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.12–7.09 (m, 6H), 7.04–6.99 (m, 12H), 4.62–6.61 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 143.7, 143.1, 140.6, 132.3, 132.2, 131.7, 131.2, 127.9, 127.7, 126.7, 126.2, 126.1, 48.4. HRMS (MALDI-TOF), *m*/*z*: 474.1454 ($[M]^+$, calcd 474.1224).

Gel electrophoresis

The protein samples were treated with denaturizing 4× sample buffer containing 62.5 mM Tris (pH 8.5), 20% (v/v) glycerol, 4% SDS (w/v), and 3% (w/v) DTT for 5 min at 85–95 °C in Protein Lobind Eppendorf cups. Serial dilutions of the proteins were labeled and loaded onto the gel lanes. Electrophoresis was carried out on self-cast polyacrylamide mini-gels (1 mm thick) using a discontinuous buffer system. The separation gel (pH 8.8) contained 12% polyacrylamide. The stacking gel (pH 6.8) contained 4% polyacrylamide. Both gels have an acrylamide/bis ratio of 37.5 : 1. The running buffer contained 25 mM Tris (pH 8.6), 192 mM glycine and 0.1% SDS (w/v) in water. All solutions were freshly prepared prior to use. SDS-PAGE was carried out on a vertical polyacrylamide gel system at a current of 15 mA until the protein bands reach the interface of the separating gel. Separation was performed at 100 V for two hours.

Fluorescent prestaining protocol

Fluorescence prestaining of proteins for SDS-PAGE gels was applied prior to the denaturation step. Dissolve ~1 mg of the protein in 1 mL of 0.1 M sodium bicarbonate buffer solution (15 μM). 10 μL of the compound **2** DMSO stock solution (5 mM) was added to 100 μL of protein solution and the pH was optimized to ~9.0 by adding 1 M sodium bicarbonate buffer. The reaction mixture was incubated for 2 h at 37 °C in an Eppendorf Thermomixer. Then, samples were cooled either to room temperature for instant use or frozen for eventual use.

Coomassie brilliant blue (CBB) poststaining and destaining protocol

2.5 g of Coomassie blue R250 was dissolved in 1000 mL of 50% (v/v) methanol, 10% (v/v) acetic acid and 40% (v/v) water with stirring as needed. The solution was filtered to remove any insoluble material. The final concentration of Coomassie blue R250 was 0.25% (w/v). After electrophoresis, the apparatus was disassembled and the gel was immersed into CBB solution. The gel was stained at room temperature overnight with gentle agitation. The Coomassie stain was removed by aspiration after staining. The gel was then immersed into the destaining solution composed of 50% (v/v) methanol, 10% (v/v) acetic acid and 40% (v/v) water which allowed the gel to destain with gentle agitation.

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The destaining step was repeated several times with removal of destaining solution at each change by aspiration. Destaining was continued until the protein bands were seen clearly without any background staining of the gel.

Fluorescent poststaining and destaining protocol

The protocol of gel fluorescent poststaining with **2** was nearly the same as that for the CBB poststaining. The only difference was the composition of the staining solution which was prepared by mixing 1 mL of DMSO stock solution of **2** (5 mM) with 20 mL of 10% SDS aqueous (w/v) solution and the pH was optimized to ~9.0 by adding 1 M sodium bicarbonate buffer. The destaining solution is 10% SDS aqueous (w/v) solution. Similar to the destaining protocol of Coomassie brilliant blue, the gel was immersed into the 10% SDS aqueous (w/v) solution and destained with gentle agitation. The destaining solution was changed every two hours and the whole destaining process was finished within six hours.

Detection of proteins

All stained gels were imaged with the Gel Doc XR+ documentation system (Bio-Rad). The images were analyzed by Quantity One gel image analysis software (Bio-Rad). The trace quantity, which represented the intensity (int) of each pixel multiplied by the band-area (mm^2), was plotted *versus* the protein amount.

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