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A highly sensitive "switch-on" fluorescent probe for protein quantification and visualization based on aggregation-induced emission†

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A highly sensitive and water-soluble "switch-on" fluorescent probe with aggregation-induced emission characteristics was developed for protein quantification and visualization. It offers a rapid, economic and effective way for the assay of complete serum proteins and disease-marker proteins.

Protein analysis is of prime importance to proteomics research that aims at interpreting biological and physiological processes at the protein level. A quantity of molecular probes for protein assays have been developed by utilizing their photophysical changes, which is caused by physical interaction or chemical reaction with proteins.[1] Colorimetry, such as Lowry and Brandford assays, is a traditional analytical method for protein quantitation in solutions.[2] When the chromophores bind with neutral organic or inorganic molecules or ions, they can exhibit a color change, which enables researchers to investigate the changes in the free guest ions or in the concentration of molecules by utilizing absorption spectroscopy.[3] However, these methods lack sensitivity and accuracy and require lengthy staining and destaining procedures. Biosensors based on fluorescent (FL) materials have been proven useful for the assay of proteins in solution and attracted wide-spread attention owing to their rapidity, low background signals and selectivity. Several FL probes that target proteins, including Nile Red, SYPRO Ruby, NanoOrange and fluorescamine, have been developed and widely used for proteomics research.[4,5] Although FL techniques offer high sensitivity, low background noise and a broad dynamic range, some FL bioprobes are insoluble in aqueous solutions, require lengthy procedures with time-sensitive steps,[6] some are unstable under ambient conditions (*e.g.*, fluorescamine and Nile Red),[7] and some are extremely expensive and sold only as a formulated solution.[4,5] These shortcomings greatly limit the scope of their real-life applications. Therefore, it is necessary for us to develop environmentally stable and synthetically readily accessible bioprobes with high sensitivity, low background signals and high selectivity.

A thorny problem encountered by conventional FL reagents in aqueous medium or physiological buffers is the ubiquitous aggregation-caused quenching (ACQ) effect,[8] which is attributed to the non-radiative relaxation of excited states of aggregates forcing researchers to study and employ them as single molecules in very dilute solutions. However, the use of dilute solution causes many problems such as weak emissions and poor sensitivity in fluorescent imaging bioassays. Even in dilute solutions, the ACQ effect can still not be avoided, because, in biological analysis, the fluorophore molecules may accumulate on the surfaces of the biomacromolecules and aggregate in the hydrophobic cavities.[9] Recently we have synthesized 9,10-bis[4-(3-sulfonatopropoxyl)-styryl]anthracene sodium salt (BSPSA, Fig. 1A) and discovered that BSPSA is not only soluble in aqueous medium but also behaves in the absolute opposite way: aggregation plays a constructive, instead of a destructive, role in the light-emitting process (aggregation-induced emission,[10] AIE) and BSPSA is found to be non-luminescent in the solution but emissive in the aggregate sate. Hence, the strong points of BSPSA prompted us to further explore its potential in bioanalysis. In this work, we investigated the performance of BSPSA as a fluorescent

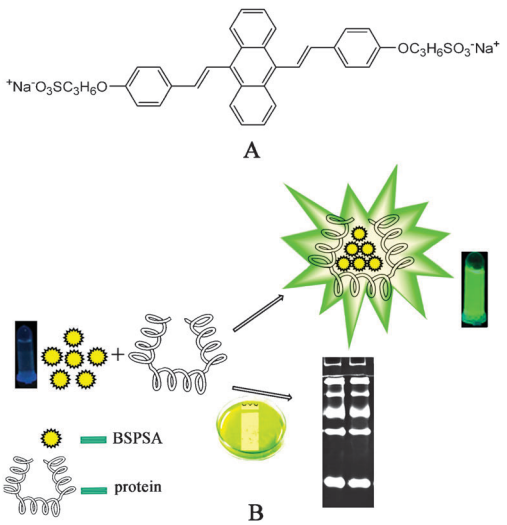

Fig. 1 (A) Chemical structure of water-soluble AIE fluorophore molecules BSPSA; (B) Illustration of the mechanism of protein visualization after PAGE using BSPSA as a fluorescent probe with AIE characteristics.

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probe for the detection and quantitation of human serum proteins. The FL "turn-on" properties of BSPSA after aggregation in the hydrophobic cavities of proteins facilitated the rapid fluorescent imaging and quantitative of complete proteins in human serum after polyacrylamide gel electrophoresis (PAGE).

In this context, BSPSA has received special attention for the following reasons: (1) BSPSA can realize fluorescent imaging of complete proteins in human serum in ten minutes, fixation and destaining procedures are not required; (2) BSPSA has high sensitivity (0.78 ng μL^{-1} of ferritin in gel), which is comparable to that of silver staining; (3) BSPSA is soluble and stable in aqueous medium, and organic solvents are not required, for this reason BSPSA is a "safe" stain. Hence, BSPSA is a good protein indicator for its rapid and sensitive detection.

The mechanism of protein visualization after PAGE using BSPSA as a fluorescent probe is schematically illustrated in Fig. 1B. The solutions of BSPSA are weakly luminescent, that is because the intramolecular rotation of these probes in solution is active and will form a relaxation channel for non-radiative decay of the excited state. When a certain amount of protein is introduced, the BSPSA may accumulate in the hydrophobic cavities or pockets of the folding structures,[11] and the intramolecular rotation of BSPSA is restricted in the aggregates due to physical constraint, which will prevent the energy from losing by non-radiative jump and activate the emission transition. As a result, the fluorescence of BSPSA would increase significantly in the presence of proteins.[12] Thus, the purpose to use BSPSA as a fluorescent probe for detecting proteins after PAGE would be achieved. The effect of increasing the concentration of HSA on the fluorescence spectra of BSPSA and the dependence of the FL intensity of BSPSA on different proteins are shown in Fig. S1 and S2, respectively (ESI†).

To demonstrate the application of BSPSA as a fluorescent probe, protein markers in the gel obtained after PAGE were stained by BSPSA. Fig. 2A shows an emissive image of a gel containing electrophoresized protein markers (for ferritin from left to right: 0.20–100 ng μL^{-1}) after staining with BSPSA (0.3 mg/100 mL) for 10 min. The lowest concentration of ferritin detected after staining with BSPSA is 0.78 ng μL^{-1} (lane 8 in Fig. 2A). Conventional protein detection methods include CBB-R250 staining, SYPRO-Ruby staining, silver staining, double-metal-chelate (DMC) staining and so on.[13,14] However, many of these methods involve careful timing and time-consuming procedures. The most frequently used and commercially available protein dyes, such as colorimetric CBB-R250 and fluorimetric SYPRO-Ruby, require lengthy staining and destaining steps. Unlike the conventional dyes, neither lengthy destaining nor careful timing procedures were required for BSPSA staining, and there was no overstaining and decrease in signal intensity after staining or destaining for 24 h. For comparison, conventional protein staining methods, including CBB-R250 staining and SYPRO-Ruby staining, are shown in Fig. 2B and C, respectively. When compared the three detection methods, the BSPSA staining shows clearer fluorescent protein bands with relatively higher resolution and signal intensity. Taking the band of ferritin for example, although we made great efforts to enhance the contrast and

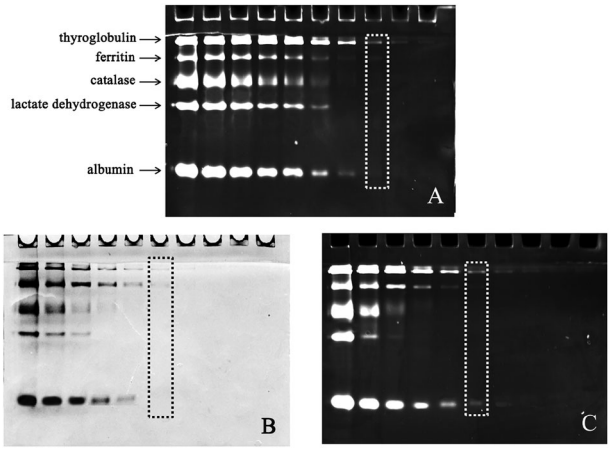
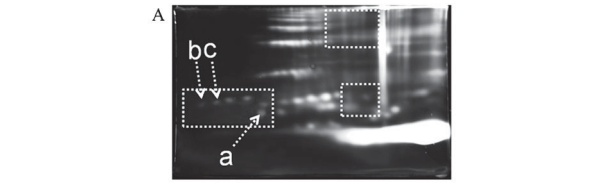


Fig. 2 Comparison of the sensitivities of (A) BSPSA staining, (B) CBB-R250 staining and (C) SYPRO-Ruby staining. Serial dilutions of marker proteins loaded in the wells (for ferritin from left to right) were (1) 100.00 ng μL^{-1}, (2) 50.00 ng μL^{-1}, (3) 25.00 ng μL^{-1}, (4) 12.50 ng μL^{-1}, (5) 6.25 ng μL^{-1}, (6) 3.13 ng μL^{-1}, (7) 1.56 ng μL^{-1}, (8) 0.78 ng μL^{-1}, (9) 0.39 ng μL^{-1}, and (10) 0.20 ng μL^{-1}, respectively.

brightness of the images that were stained by conventional methods, it can only be recorded in lane 6 for SYPRO-Ruby staining and CBB-R250 staining. As indicated, the sensitivity of BSPSA-based fluorescent imaging is higher than the two conventional methods.

In order to evaluate the performance of BSPSA in clinical applications, we applied BSPSA in the detection of human serum proteins. Because of the high resolution and high quantity of proteome information, native 2D-PAGE was introduced to examine the applicability of BSPSA for detecting human serum samples. Three gels were electrophoresed in parallel and the proteins after 2D-PAGE were stained with BSPSA, CBB-R250, and silver respectively. When comparing the results of the three different staining methods, the proteins marked in the rectangle regions could be hardly detected with the conventional methods. However, because of the high FL intensity of proteins and low background in BSPSA-based fluorescent imaging, several new protein spots marked in Fig. 3A have been observed. In addition, we also detected the human serum proteins after native 1D-PAGE with BSPSA. Compared with conventional methods, BSPSA-based fluorescent imaging offers clearer protein bands, stronger signals, lower backgrounds (Fig. S6†), and the detection limit for tranferrin is comparable to that of the most sensitive silver staining (Fig. S7†). As shown in Fig. S8,† a linear relationship is obtained between the relative fluorescent intensity of the scanned bands in the gel (transferrin) and the protein concentration, and the linear dynamic range for BSPSA-based fluorescent imaging is 5.5–55 ng μL^{-1} ($R^2 = 0.998$), which is the same as silver staining. Thus, BSPSA shows great potential as an excellent dye for the detection of complete proteins.

To identify the proteins only detected by BSPSA-based fluorescent imaging, three protein spots indicated by the dotted arrows on the gel image in Fig. 3A were identified using MS/MS techniques. The results are shown in Fig. 3D. From the results of the peptide mass data, spots a, b, and c were zinc-α_2-glycoprotein (ZAG), thyroxine-binding globulin



D

Spot No.	Protein name	Accession	Mass (Da)	Peptides	Scores
a	Zinc-alpha-2-glycoprotein	IPI00166729.4	34237	25	125
b	Thyroxine-binding globulin	IPI00292946.1	46295	10	50
c	Alpha-2-HS-glycoprotein	IPI00953689.1	39300	12	68

Fig. 3 The detection of human serum after 2D-PAGE. (A) BSPSA-based fluorescent imaging, (B) CBB-R250 staining, (C) silver staining, and (D) proteins identified by MS/MS.

(TBG) and α_2-HS-glycoprotein (AHSG), respectively. Zinc-α_2-glycoprotein (ZAG), secreted from the liver and adipose tissues, is a 40 kDa glycoprotein with a serum level of 27.4 ± 8.3 μg mL^{-1}.[15] ZAG holds great interest as it plays many important functions in physio-pathological processes, including the regulation of melanin production by melanocytes and prostate and bladder cancers. Thyroxine-binding globulin (TBG), a 58 kDa single-chain polypeptide glycoprotein secreted from the liver, is the major thyroid-hormone transport protein in the serum, and is consequently significant in the regulation of thyroid hormone levels in plasma.[16] The serum level of TBG in normal adults ranges from 20 to 48 μg mL^{-1}, with a mean level of 36. Alpha2-HS-glycoprotein (AHSG), a 45 kDa glycoprotein synthesized by hepatocytes, plays an important role in the regulation of lipid transport, bone mineralization, and a decreased AHSG level in serum is associated with infection, malignancy and inflammation.[17] Thus, the BSPSA-based fluorescent imaging has shown its great potential in clinical diagnosis.

In this work, a novel, synthetically readily accessible and environmentally stable AIE bioprobe has been developed for the detection and quantitation of human serum proteins. BSPSA is successfully used for protein staining after PAGE. The detection limit of BSPSA-based fluorescent imaging for ferritin in gel is 0.78 ng μL^{-1}, which was much lower than that of CBB-R250 staining and SYPRO-Ruby staining. In comparison with conventional protein dyes, BSPSA-based fluorescent imaging showed great potential in protein assays because of its simple and rapid staining procedure, high sensitivity, low detection limits and low background. In addition, we have demonstrated that several relatively low-abundance proteins in serum can be easily detected with the proposed method.

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