Protein Detection

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A Protein-Responsive Chromophore Based on Squaraine and Its Application to Visual Protein Detection on a Gel for SDS-PAGE

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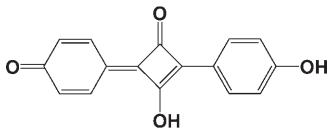
As proteomic analysis has become an important field for assessing the techniques that generate proteomic data, biochemists are searching for various ways to detect proteins with easy handling, rapid monitoring, high sensitivity, and good binding linearity for both qualitative and quantitative analyses.[1] Several detection methods are suitable for the development of sensitive protein quantification in solution or gel for electrophoresis: 1) absorption spectrometry for the detection of tryptophan or tyrosine in proteins, 2) fluorescence spectrometry,[2] and 3) colorimetry.[3] Colorimetry is a traditional analytical method, and chromophores responsible for a color change upon binding ions or neutral organic or inorganic molecules have enabled researchers to investigate the changes in the free guest ions or concentrations of molecules by means of absorption spectroscopy, reflectance spectroscopy, and paper sensing using dry chemistry.[4] The Bradford assay is one of most widely used colorimetric methods for the detection of proteins in solution.[3] The Bradford assay uses Coomassie Brilliant Blue (CBB) as the protein-binding dye. The binding of CBB to a protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, together with an increase in the absorbance at 595 nm. This assay is easy to conduct and rapid, and the dye-binding process is virtually complete. However, this assay has a large protein-to-protein variation, and detergents such as sodium dodecyl sulfate, Triton X-100, and commercial glassware detergents produce an interference with the protein assay.

We considered several requirements for developing a colorimetric reagent for proteins: 1) a higher molar extinction coefficient which may guarantee the use of lower dye concentrations, 2) reduced interference from foreign substances, and 3) noncovalent interactions, such as a hydrophobic interaction or an electrostatic interaction, which produce a rapid reaction between the protein and dye.

Molecules that are solvatochromic are sensitive to changes in the external environment, and their absorption spectra generally respond to the environment near the dye, resulting in dramatic changes in the absorption spectra and color.[5] Several solvatochromic dyes have been designed and synthesized, and they find application in ion and molecular sensors, electrophotography, solar cells, and nonlinear optical materials.[6] Our group screened various types of solvatochromic dyes for the protein assay and discovered that the squaraine dye **1**, which bears a hydroxy group, undergoes a



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color change upon binding to proteins in either solution or gel for electrophoresis. The experimental results clearly showed that the squaraine dye has excellent protein sensitivity, is easy to handle, has a short reaction time, and can be used for protein staining in the gel after SDS-PAGE.

To study the in vitro photophysical properties of the protein indicator, compound **1**, we recorded its absorption spectrum in a buffer solution of pH 7.0 at 25 °C. Figure 1a indicates the typical changes in the absorption spectrum of compound **1** in the presence of various concentrations of bovine serum albumin (BSA). A solution of compound **1** alone has pink color and the molar extinction coefficient (ε) is 5000 at 600 nm (Figure 1b). In contrast, the **1**·BSA complex forms a deep purple solution. This is an obvious color change for compound **1** upon complex formation and corresponds to a dramatic increase in the absorbance at around 600 nm (Figure 1a). The ε value of the **1**·BSA complex is 84000 at 600 nm, which is about 17 times higher than that of compound **1** alone.

These spectral changes are caused by a solvatochromic process in compound **1** upon binding to protein. It is known that the squaraine part in compound **1** induces a very large ground-state dipole moment, which decreases significantly upon excitation because of intramolecular charge transfer.[6b] The difference between the ground and the excited state depends on the external environment of compound **1** which includes effects such as solvent polarity and the desolvation process. Compound **1** is strongly bound at the hydrophobic positions of the BSA,[7] and the solvatochromism of compound **1** is caused by the desolvation process, which creates

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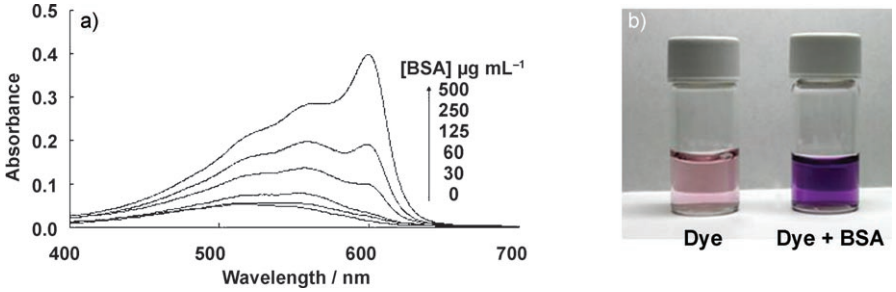

Figure 1. a) Absorption spectra of compound **1** before and after the addition of BSA in concentrations ranging from 0 to 500 μg mL^{-1}. b) Photographs of solutions of compound **1** in the absence and the presence of BSA.

the dramatic change of molar extinction coefficient which responds linearly to the amount of BSA in solution.

The absorbance of compound **1** at 600 nm was plotted as a function of BSA concentration, and a typical calibration graph of the response to BSA under the optimum experimental conditions was obtained (Figure 2). This plot shows a

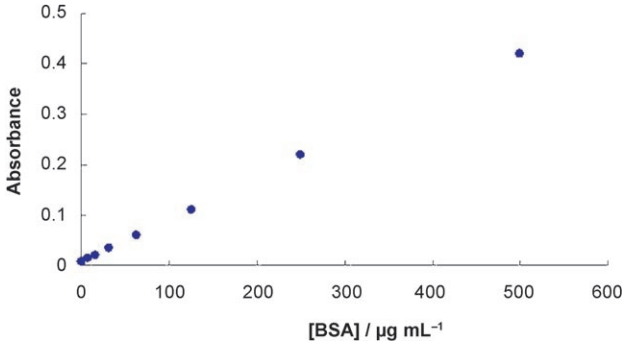

Figure 2. Plot of the absorbance of **1**·BSA at 600 nm as a function of BSA concentration.

good linear relationship between the absorbance and the BSA concentration ($r^2 > 0.998$) up to 500 μg mL^{-1} of BSA. The detection limit is 2 μg mL^{-1} for the BSA (signal to noise ratio was 3.0), which is five times higher than that of Coomassie Brilliant Blue (CBB).

The conventional methods of total protein detection, such as Bradford assay and Lowry assay, exhibit various responses to different proteins, which are related to the amino acid sequence, pI, protein structure, and the presence of certain side chains that can dramatically alter the protein's response. In order to observe the protein-to-protein variability of compound **1**, the absorption spectral changes of compound **1** were monitored in the presence of proteins besides BSA, such as IgG and BGG. The data are shown in the Supporting Information. We found that compound **1** indicates almost the same response to all three proteins tested. The protein-to-protein variations of compound **1** were compared to the commercially available protein-detecting reagents CBB and WST-1.[8] CBB and WST-1, which are colorimetric reagents for the detection of proteins, are affected by the different proteins and show large protein-to-protein variations. In comparison, compound **1** is not affected by the different proteins and indicates the same response against the various proteins. Based on these results, compound **1** has the same response even if the structure of the protein is different, and it is possible to monitor the correct protein concentration using only one calibration graph.

The response of compound **1** to various contaminants, such as inorganic salts, detergents, chelating reagents, thiol compounds, reductants, was tested to investigate the interference of these nonprotein substances in protein analysis. All the tests were carried out at a concentration of 1000 μg mL^{-1} of BSA mixed with 5.0 μM of compound **1** in the presence of an excess amount of the foreign substances. Details on the foreign substances and the maximum concentrations that produce absorbance perturbation less than 10% are given in the Supporting Information. We found that the response of compound **1** to BSA, that is, the formation of a stable **1**·BSA complex, is insensitive to the excess amount of nonprotein substances. Unlike the Lowry and BCA assays, the assay using compound **1** is compatible with the presence of reducing agents, and the nucleic acid does not interfere with the protein quantification. Compound **1** produces the same response before and after the addition of these substances, thus making it possible to monitor the correct protein concentration without any interference from a foreign substance.

To demonstrate the application of compound **1**, proteins in the gel obtained after the electrophoresis using 1D SDS-PAGE minigels were stained by compound **1**. After fixation of the proteins, the gel was incubated in the staining solution and washed with the solution containing H_2O/MeOH/AcOH 87:10:3 v/v. Figure 3 shows the typical gel images of BSA

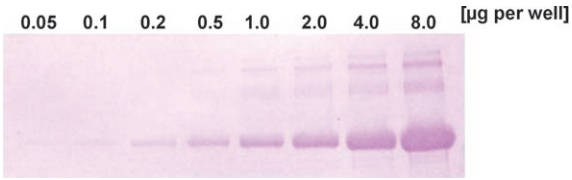

Figure 3. Typical gel image of BSA stained by compound **1**.

and sheep IgG samples after staining with compound **1**. Compound **1** is bound to the BSA samples by a stable interaction, and purple spots of BSA in the gel were observed. The compound **1** staining indicated a good linear relationship between the integrated volume of the densitometry units for scanned bands of proteins in the gel and protein concentration ($r^2 > 0.996$). The detection limit was 30 ng per well for BSA (signal-to-noise ratio was 3.0). To examine the reproducibility of the calibration graph, the reproducibility tests ($n = 3$) were carried out, and the relative standard deviation of the

response was within 2.0%. Coomassie brilliant blue staining is one of the common methods for the universal profiling of proteins in gels, and the protein spots in gel are detected visually. Although CBB staining is an inexpensive method, the detection limit for BSA is 64 ng per well, and it requires destaining and is time consuming (total time for staining is 105 min).[9] In contrast, the staining procedure of compound **1** requires 60 min, and the detection limit for this method is about two times higher than that of the CBB staining method. From this viewpoint, our method is a highly sensitive and speedy staining protocol for the visual colorimetry of proteins in the gel for electrophoresis.

The present study described the discovery of a protein-binding indicator which noncovalently interacts with proteins and provides a dramatic color change from orange to deep purple upon binding to proteins. This indicator responds to various proteins, and the detection of the proteins is not affected by the presence of contaminants. In addition, protein staining on 1D SDS-PAGE minigels was successful, had high sensitivity, and was easy to conduct. We are currently investigating other protein indicators based on the results of this study.

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