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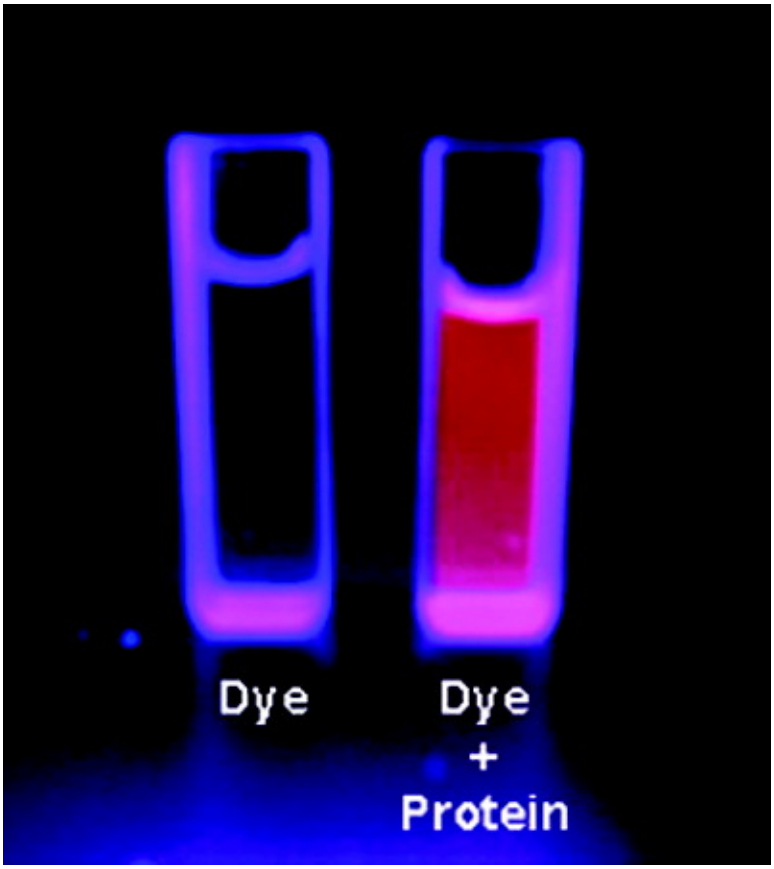
J. Am. Chem. Soc., **2005**, 127 (50), 17799-17802• DOI: 10.1021/ja054739q • Publication Date (Web): 22 November 2005



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Design and Synthesis of Intramolecular Charge Transfer-Based Fluorescent Reagents for the Highly-Sensitive Detection of Proteins

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Abstract: Novel fluorescent molecular probes possessing both a hydroxystyryl and a cyanopyranyl moieties were designed and synthesized to detect the proteins via noncovalent bonding. These fluorescent probes indicated very weak fluorescence emission in the absence of protein. On the other hand, the fluorescence spectra of these probes showed a large Stokes shift and dramatic increase of fluorescence intensity, and red emission was observed after addition of BSA. These fluorescence spectral changes upon binding proteins were caused by the ICT process. Fluorescence intensities of the probes were plotted as a function of protein concentrations. A good linear relationship was observed up to 1000 μg/mL of protein, and the detection limit was found to be 100 ng/mL at the given assay conditions. Similar results were observed for the measurements of not only BSA but also other proteins (BGG, etc.). The responses of these probes to various nonprotein substances (inorganic salts, chelating agents, etc.) were observed, the fluorescence intensity did not change before and after the addition of foreign substances, and correct protein monitoring was successful using these fluorescent probes. To demonstrate the application of these probes, proteins after the separation using SDS-PAGE were stained in the medium containing **1**, and the imaging of the proteins in the gel was successful. The experimental results clearly showed that these probes are good protein indicators for easy and highly sensitive detection.

Introduction

Protein biochemists are searching for various ways to detect proteins with high sensitivity and good binding linearity to facilitate both qualitative and quantitative analysis. For developing sensitive protein quantification in solution or in gel,there are several methods to detect proteins as follows: (i) absorption spectrometry, (ii) Biuret method, (iii) Lowry method, (iv) Bradford method, and (v) fluorescence spectrometry. Fluorescence spectrometry is a conventional and highly sensitive analytical method, and fluorescent probes that indicate a spectral response 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 fluorescent microscopy, flow cytometry, and fluorescent spectroscopy. Several fluorescent reagents targeting proteins have been developed for the detection of proteins in solution, such as fluorescamine and cyanine dyes[1,2] and in SDS−polyacrylamide gels, such as SYPRO Ruby.[3] Fluorescamine does not produce a fluorescence emission, whereas a strong green fluorescence at 495 nm was observed when excited at 395 nm after the reaction with the primary amine in the protein. Hydrophobic cyanine dyes indicate an increase in the emission intensities upon binding to protein−sodium dodecyl sulfate (SDS) complexes. However, these reagents used for the fluorescence spectrometry and for the other analytical methods have some disadvantages during the protein measurement that include (i) long reaction time, (ii) aggregation of dyes, (iii) small Stokes shift, and (iv) nonlinear and sigmoidal calibration curve.[4] SYPRO Ruby is the highly sensitive fluorescent dye targeting for proteins in SDS−polyacrylamide gels, whereas this dye is not able to be stained in the presence of SDS, and it takes a long time to eliminate the SDS from the gels completely and to remove the excess dyes after staining.

We considered several requirements when designing a fluorescent probe for proteins (i) efficient excitation with most laser-based instrumentation, (ii) reduced interference from foreign substances, (iii) higher molar extinction coefficient and quantum yield, which may guarantee the use of lower dye concentrations, (iv) noncovalent interaction, such as a hydrophobic interaction or an electrostatic interaction, and (v) elimination of the need for washing to remove SDS and excess dyes in the staining protocols.

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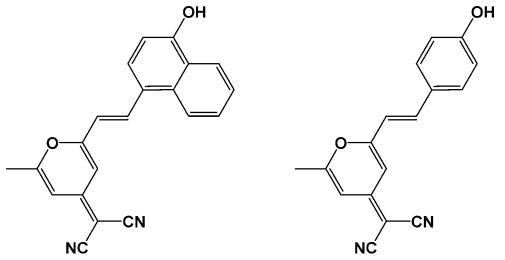

1 2

Figure 1. Chemical structures of fluorescent protein indicators, **1** (2-{2-[2-(4-hydroxynaphthalen-1-yl)-vinyl]-6-methylpyran-4-ylidene}-malononitrile) and **2** (2-{2-[2-(hydroxyl-phenyl)vinyl]-6-methylpyran-4-ylidene}malononitrile).

Intramolecular charge transfer (ICT) molecules are sensitive to changes in the external environment, and their quantum yields generally increase as the environment becomes hydrophobic, which produces the dramatic fluorescence spectral changes.[5,6] 4-(Dicyanomethylene)-2-methyl-6-(*p*-methylaminostyryl)-4H-pyran (DCM), which is a laser dye, produces ultrafast process of ICT, and the excited-state dipole moment (26.3 D) of DCM is higher than that in the ground state (5.6 D).[7−10] To take advantage of this phenomenon, our group designed and synthesized novel fluorescent probes, which undergo an ICT process and whose quantum yields linearly respond to the amount of proteins in solution by interacting with the proteins in this study. Figure 1 shows the chemical structures of the fluorescent probes, whose molecular structures were confirmed by their spectroscopic data. Additionally, an aromatic hydrocarbon possessing a hydroxyl group was incorporated into the probes via double bonds to enhance the hydrophobic interaction with proteins, to make them water soluble, and to produce a conjugated π-electron system.

Methods

To study the in vitro photophysical properties of protein indicators, **1** and **2**, the excitation and emission spectral measurements were obtained in a buffer solution of pH 7.0 at 25 °C. Figure 2a indicates the typical excitation and emission spectra of **1** in the presence of 1000 μg/mL of bovine serum albumin (BSA). The **1**·BSA complex showed an excitation maximum at 582 nm which was monitored at 650 nm. However, the actual excitation wavelength was set for 550 nm, because fluorescence spectrometer using for this study scans the emission wavelength up to 750 nm, which restricted the monitoring for wide range of emission wavelength. The compound **2**·BSA complex indicated an excitation maximum at 480 nm monitored at 540 nm. As a result, these wavelengths in the excitation spectra were used as the excitation wavelength for the optimum experimental conditions.

Results and Discussion

Figure 2b shows the fluorescence spectral changes of **1** before and after the addition of BSA. Compound **1** itself had a very weak emission, whereas the emission of the **1**·BSA complex indicated a large stokes shift and dramatic increase in the fluorescence intensity centered at around 660 nm, the photograph of which is shown in Figure 2d. The fluorescence quantum yields of **1** itself were less than 0.001; however, those of the **1**·BSA complex were 0.31. Similar results were obtained in the case of compound **2** after the addition of BSA, and an emission maximum was observed at 540 nm after the addition of BSA.

These spectral changes were caused by a complex formation, in which **1** is strongly bound at the hydrophobic positions of the BSA supported by the hydrophobic interaction. This interaction undergoes the ICT process, the quantum yields of which linearly respond to the amount of BSA in solution.

The emission intensities at 650 nm for **1** were plotted as a function of the BSA concentration, and a typical calibration graph of the response to BSA under the optimum experimental conditions was obtained as shown in Figure 2c. This plot shows a good linear relationship between the emission intensities and the BSA concentration ($r > 0.996$) up to 1000 μg/mL of BSA. The detection limit was 100 ng/mL of BSA ppm (signal-to-noise ratio was 3.0).

The conventional total protein detections exhibit various responses for different proteins. These differences 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. Most of the protein detection methods use BSA or IgG as the standard samples to create a calibration graph, and then the concentration of the protein in the sample is determined. Table 1 indicates the protein-to-protein variation using **1** in fluorescence response, which was obtained by the following equation: ratio = (slope of various proteins)/(slope of BSA). The response for BSA was normalized to 1.00. **1** indicated the same response to different proteins. The protein-to-protein variations of **1** were compared with the commercially available protein-detecting reagents (CBB and WST-1).[11] CBB and WST-1 were affected by the different proteins and, in particular, WST-1 was significantly affected by the proteins. On the other hand, compound **1** was not affected by the different proteins and indicated the same response against the various proteins. On the basis of this result, we determined that this indicator 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 responses of **1** to various nonprotein substances (inorganic salts, detergents, chelating reagents, thiol compounds, reductants, etc.) were tested to investigate the interference of these nonprotein substances with the correct protein estimation. All the tests were carried out at a concentration of 1000 μg/mL of BSA mixed with 1.0 μM of **1** in the presence of an excess amount of foreign substances. The detailed foreign substances and the maximum concentrations which give the perturbation of fluorescence intensity less than 10% can be obtained from the Supporting Information. From the result, the response of **1** to BSA was not affected by the excess amount of nonprotein substances. In particular, it is known that most ionic and nonionic detergents interfere with the correct protein detection due to the reduction of the dye·protein complex and to the precipitation of the assay reagent. The response of **1** against BSA was affected by the detergents at the concentration of 1.0%

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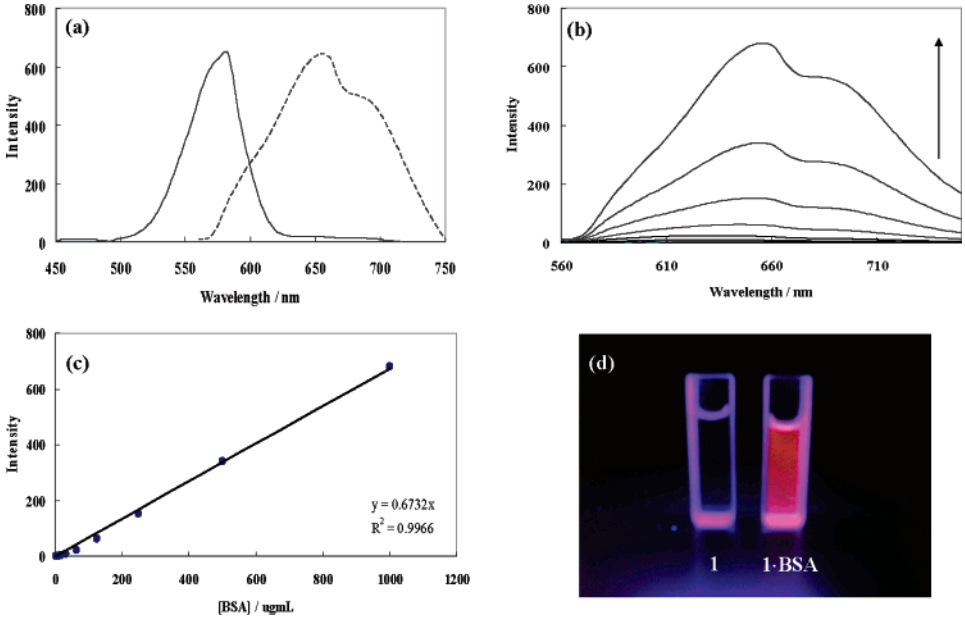

Figure 2. Excitation spectrum (solid line) and fluorescence spectrum (dashed line) of compound **1** in the presence of 1000 μg/mL of BSA (a), fluorescence spectra of compound **1** before and after the addition of BSA from 0 to 1000 μg/mL (b), plot of the fluorescence intensity at 660 nm as a function of BSA concentration (c), and photographs of compound 1 solutions in the absence and the presence of BSA (d). HEPES, 20.0 mM (pH 7.2). Excitation at 550 nm for the fluorescence measurements.

Table 1. Protein-to-Protein Variation of **1**

protein	protein vs BSA
BSA	1.00
BGG	0.86
transferrin	0.92
rabbit IgG	0.90
chymotrypsinogen A	1.02
bovine IgG	0.88
sheep IgG	0.96
human IgG	1.02

for SDS, 0.8% for CHAPS, 0.5% for Tween 20, 1.0% for Triton X-100, respectively. However, these high concentrations of detergents are usually not used for the measurements of proteins, and the precipitation of **1** did not happen under these conditions. To take advantage of these phenomena, this indicator produced the same response before and after the additions of these substances, thus making it possible to monitor the correct protein concentration without any interference from a foreign sub stance.

To demonstrate the application of these new protein fluorescent probes, proteins after electrophoresis using 1-D SDS-PAGE minigels were stained by **1** and were scanned using the image analysis systems. Figure 3 shows the gel images of BSA and sheep IgG samples after **1** staining. The association of **1** with these proteins and a visual examination of the staining response were successful. Commercially available fluorescent dyes such as SYPRO Ruby[12] need a long time for staining (overnight) and for washing the gels to remove SDS and excess dyes completely (60 min). On the other hand, the staining procedure of compound **1** required only 30 min and fluorescent image of proteins was observed despite the removing of SDS and excess dyes in the gel, which makes it possible to produce an easy handling and to rapidly obtain the results.

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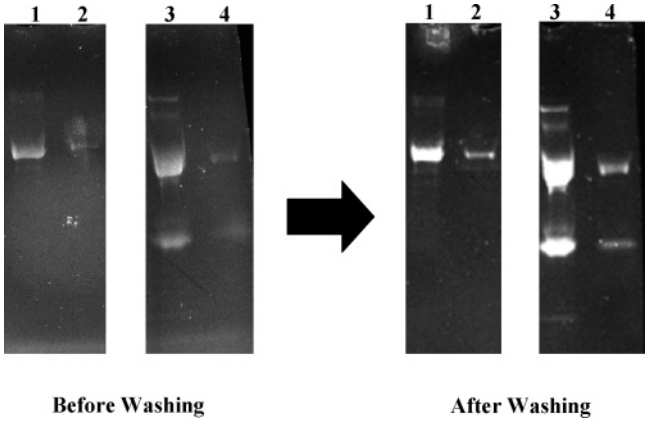

Figure 3. Staining of BSA and IgG with **1** dye by SDS-PAGE, applied to each gel patch: (1) BSA 2.0 μg, (2) BSA 0.2 μg, (3) IgG 5.0 μg, and (4) IgG 0.5 μg before and after washing.

Conclusions

The present study demonstrates novel protein-binding fluorophores which interact noncovalently with proteins and provide a dramatic increase in the fluorescence intensity response to proteins. The photophysical properties indicated high extinction coefficients, high quantum yields, and large stokes shifts. These probes responded to various proteins, and the detection of the proteins was not interfered with by the presence of foreign substances (nonprotein compounds). In addition, the successful demonstration of fluorescent staining on 1-D SDS-PAGE minigels was performed using the fluorescent probe **1**. Therefore, these new fluorescent molecular probes will be used to investigate proteins. We now expect to carry out the very short time and very easy gel electrophoresis using these probes. Moreover, we are currently designing other protein indicators based on this unique molecular design

Acknowledgment. This work is financially supported by High-Throughput Biomolecule Analysis System Project by New Energy and Industrial Technology Development Organization (NEDO), Japan.

Supporting Information Available: Detailed synthetic procedures of these probes. This material is available free of charge via the Internet at http://pubs.acs.org.

JA054739Q