Fluorescent Hydrophobic Probes Based on Intramolecular Charge Transfer State for Sensitive Protein Detection in Solution

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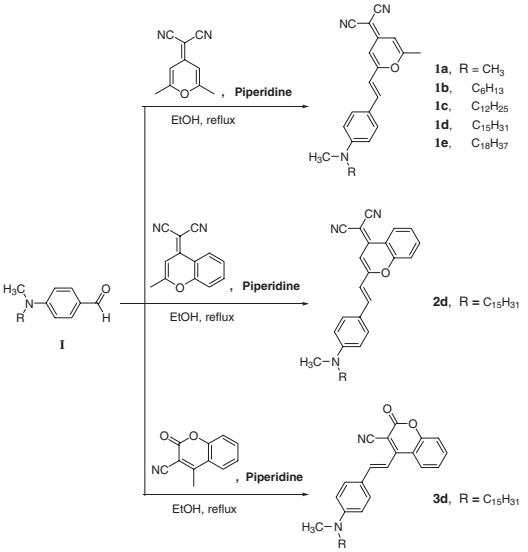
New hydrophobic intramolecular charge transfer probes, comprising an aminostyryl and a cyanopyranyl moieties, were developed for sensitive protein quantification in solution. The probes exhibited dramatic increase of fluorescence quantum yields with accompanying blue-shift of the emission when bound to protein–sodium dodecyl sulfate (SDS) complexes. Using this emission enhancement, the synthesized probes were successfully applied for determining protein concentrations in solution.

For developing sensitive fluorescent protein quantification reagent in solution, much of the effort has been directed to hydrophobic cyanine dyes whose emission intensities increase upon binding to protein–sodium dodecyl sulfate (SDS) complexes.[1,2] However, hydrophobic cyanine dyes were reported to aggregate easily even in highly diluted solutions. The aggregation could be facilitated further by a high local dye concentration when dye-to-protein ratio is large.[3] As a result, the calibration curve of a commercial protein quantification reagent, NanoOrange® (Molecular Probes, Inc.), fits to a non-linear sigmoidal curve.[1]

Fluorescence spectra of intramolecular charge transfer (ICT) molecules are sensitive to their environment[4] and their quantum yields generally increase as the environment becomes hydrophobic.[5] In the present study, we explored non-cyanine fluorescent probes, which undergo ICT process and whose quantum yields respond linearly to the amount of proteins in solution by interacting with hydrophobic SDS molecules associated with proteins. We used a well-known 4-(dicyanomethylene)-2-methyl-6-(*p*-methylaminostyryl)-4*H*-pyran (DCM) that transforms to an ICT state upon excitation, and its analogs as fluorophores.[6] Additionally, a hydrocarbon tail ($-(CH_2)_nCH_3$, $n = 0$, 5, 11, 14, 17) was incorporated into the probes to enhance hydrophobic interaction of the probes with the SDS molecules. Scheme 1 depicts the structures and synthesis of the fluorescent probes, whose molecular structures were confirmed by their spectroscopic data.[7]



Scheme 1. Structures and synthesis of fluorescent probes.

All probes exhibited a broad red emission centered at around 610–670 nm (Figure 1). Their fluorescence spectra were highly sensitive to solvent polarity, showing a gradual red-shift of the emission as the solvent polarity increased (inset of Figure 1).[8] Quantum yields of all probes in ethanol were high in the range of 0.1–0.4 except that of **3d**, which was only 0.02.

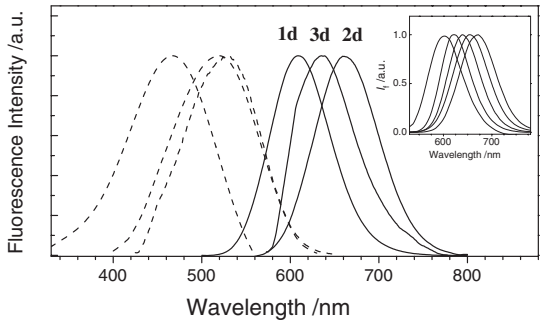


Figure 1. Normalized fluorescence excitation (dashed line) and emission (solid line) spectra of the synthesized probes in ethanol, **1d**, **3d**, and **2d** from the left, respectively. Inset depicts the normalized fluorescence emission spectra of **2d** in toluene, chloroform, tetrahydrofuran, iso-propyl alcohol, and ethanol from the left. [**2d**] = 1 μM.

Applicability of the synthesized fluorescent probes as protein detection reagent in solution was examined by measuring emission spectra of the solutions with various amounts of bovine serum albumin (BSA) while keeping the SDS concentration constant. As shown in Figure 2, the emission intensity of **1c** increased dramatically by the presence of BSA in SDS solution. Furthermore, the intensity increased progressively as the BSA concentration increased. When the dye was added to the solution containing either proteins or SDS molecules alone, little change of emission was observed (data not shown). Hence, the observed large emission increase in the solution must be the result of interactions between the probes and protein–SDS complexes. The emission increase depended on solution pH, exhibiting the maximum enhancement at pH 2–4. At higher pHs, the emission in the blank sample, containing only SDS, was increased, which

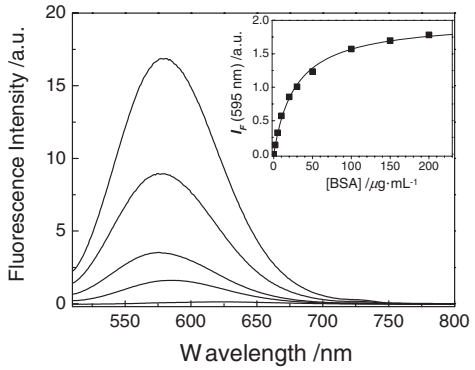


Figure 2. Emission spectra of **1c** in the presence of various amounts of BSA. BSA concentrations were 0, 1, 2, 5, and 10 μg/mL from the bottom while SDS concentration was kept constant as 0.005% in 1.5% acetic acid solution. Protein–SDS complex solutions were made by diluting 4 mg/mL BSA stock solution in 1% SDS, which was boiled for 10 min at 95 °C for complete denaturation of the protein. Inset shows the titration curve of **1c** with respect to BSA concentration. [**1c**] = 1 μM. K_d = 30 μg/mL.

Table 1. Fluorescence characteristics of the synthesized probes

Probe [a]	Buffer		BSA/SDS		
	λ_{em}/nm	I_F [b]	λ_{em}/nm [c]	I_F [c]	Q_f [d]
1a	624	1.0	620	5.1	0.12
1b	623	1.6	600	25	0.28
1c	619	2.3	595	50	0.31
1d	613	3.2	595	54	0.40
1e	612	2.5	595	60	0.34
2d	676	1.7	636	5.5	0.15
3d	668	0.7	610	2.4	0.10

[a]The probe concentration was 1 μM in 1.5% acetic acid solution. [b]Relative fluorescence emission intensities at their maxima. [c]The protein and SDS concentrations are 1 μg/mL and 0.005%, respectively, in 1.5% acetic acid. [d]Fluorescence quantum yields of the probes in 350 μg/mL BSA solution with 0.05% SDS in 1.5% acetic acid. The fluorescence quantum yields of the probes are less than 0.001 in 1.5% acetic acid.

resulted in reduction of the protein detection sensitivity. It should be noted that we observed similar changes in emission spectra with all probes. However, the extent of intensity enhancement and wavelength shift of **1a** was much less than the ones that possess a longer aliphatic chain. Apparent dissociation constant of **1c** to protein–SDS complex, K_d, was successfully estimated from the titration curve (inset of Figure 2) as 30 μg/mL for BSA when 1 μM of **1c** was used. Quantitative comparison of emission intensities of the probes responding to the presence of proteins in the solution is summarized in Table 1.

When emission intensities of **1c** were plotted as a function of protein concentration (Figure 3), an excellent linear relationship was observed ($r > 0.999$) up to 10 μg/mL protein concentration. This remarkably linear response is attributed to relatively low binding affinity of the dyes to protein–SDS complexes in combination with a large quantum yield improvement upon binding. Compared to previously reported cyanine dyes,[1,2] which contain a permanent positive charge, probes **1c**–**1e** have no permanent charge. Removing a positive charge appears to reduce binding affinity to SDS–proteins as well as dye aggregations in a great extent. The detection limit was found to be 10 ng/mL at the given assay condition. Such a high sensitivity, which is hundreds times more sensitive than conventional Bradford assay,[9] can

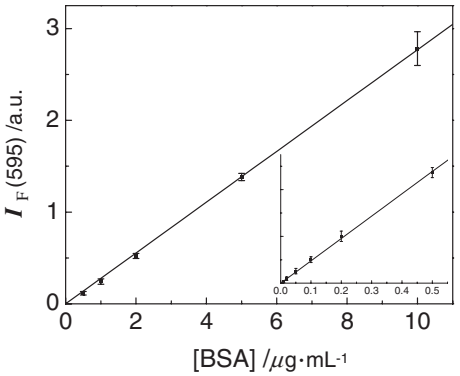


Figure 3. Calibration curve of the fluorescence emission intensity of **1c** at 595 nm as a function of protein concentration. Inset shows the region where BSA concentrations are low. Error bars represent the standard deviation of triplicate measurements.

be achieved only when the emission enhancement is extremely large. We also obtained similar linear calibration curves with **1d** and **1e**, however, **1c** seems to be more preferable among the synthesized probes considering sensitivity, solubility, and reproducibility. Furthermore, the present method successfully measured protein concentrations of rat serum samples, which were made by diluting the original serum samples by the factor of 10^4. The dilution yielded the protein concentration to be within the linear dynamic range of the present method while reducing the interferences from non-protein substances to be negligible.

In summary, we presented new ICT-based fluorescent probes and their utilization as a sensitive protein-quantifying reagent in solution, with an excellent linear response.

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7 Selected data for **1a**: HRMS (FAB) for $C_{19}H_{17}N_3O$ Calcd 304.1372 (MH^+), Found: 304.1362; Anal. Calcd for $C_{19}H_{17}N_3O$: C, 75.23; H, 5.65; N, 13.85. Found C, 75.45; H, 5.86; N, 13.83%. **1b**: HRMS (FAB) for $C_{24}H_{27}N_3O$ Calcd: 374.2154 (MH^+), Found: 373.2147. **1c**: HRMS (FAB) for $C_{30}H_{39}N_3O$ Calcd: 458.3171 (MH^+) Found: 458.3183; Anal. Calcd for $C_{30}H_{39}N_3O$: C, 78.73; H, 8.59; N, 9.18. Found C, 78.67; H, 8.60; N, 9.38%. **1d**: HRMS (FAB) for $C_{33}H_{45}N_3O$ Calcd: 500.3563 (MH^+) Found: 500.3633. **1e**: HRMS (FAB) for $C_{36}H_{51}N_3O$ Calcd: 542.4032 (MH^+) Found: 542.4089. **2d**: HRMS (FAB) for $C_{36}H_{45}N_3O$ Calcd: 536.3563 (MH^+) Found: 536.3655; Anal. Calcd for $C_{36}H_{51}N_3O$ C, 80.70; H, 8.47; N, 7.84. Found C, 80.96; H, 8.61; N, 8.02%. **3d**: HRMS (FAB) for $C_{34}H_{44}N_2O_2$ Calcd: 513.3403 (MH^+) Found: 513.3474; Anal. Calcd for $C_{36}H_{51}N_3O$ C, 79.65; H, 8.65; N, 5.46. Found C, 78.74; H, 8.80; N, 5.47%.
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