New Fluorescent Stains for Protein Detection in Sodium Dodecyl Sulfate–Polyacrylamide Gels

Soo Yeon Hong, Hyunsook Jun, Seung Soo Yoon,* Chulhun Kang,† and Myungkoo Suh*
Department of Chemistry, Sung Kyun Kwan University, Suwon 440-746, Korea
†*Department of Neuroscience, Graduate School of East-West Medical School, Kyung Hee University, Yongin 449-701, Korea*

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Highly sensitive new fluorescent stains for proteins in sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) were developed by combining an environment sensitive fluorophore and a hydrocarbon tail for improved binding to SDS molecules associated with the proteins. The experimental results showed that these dyes bound to protein–SDS complexes with a concomitant emission increase upon binding.

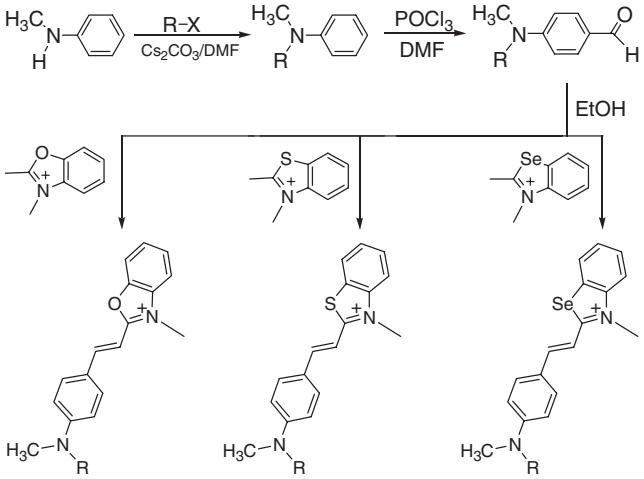
Fluorescent dyes are increasingly used for visualization of proteins in sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) due to their high sensitivity and ease of use.[1] Unlike covalent modifiers, fluorescent stains which bind non-covalently to proteins or SDS molecules associated with proteins are particularly useful for proteomic studies.[2] These non-covalent binders do not interfere post-gel microanalysis of the visualized proteins by means of mass spectrometry, which is essential for protein identification nowadays.

Various environment-sensitive fluorescent dyes were reported to increase emission intensity in the presence of hydrophobic protein–SDS complexes, and used as a protein detection reagent in gels and/or in solutions. A hydrophobic probe, 1-anilinonaphthalene-8-sulfonate (ANS) was first applied for protein staining[3] and later Nile red was used as a protein detection reagent for SDS–PAGE and for solutions with much improved sensitivity.[4] More recently, new dyes, SYPRO® Orange, Red, and Tangerine, which exhibit intense fluorescence upon binding to protein–SDS complexes, were developed as protein stains for SDS–PAGE.[5] These dyes can visualize protein bands which contain as low as 4–10 nanogram proteins.

In the present study, we developed new fluorescent dyes, which are to be used as sensitive non-covalent fluorescent stains for proteins in SDS–PAGE. The synthesized stains (**1**, **2**, and **3** in Scheme 1) contain a medium-sensitive fluorophore, which has an electron accepting group and an electron donating amino group, and a long hydrocarbon tail for increasing affinity to SDS molecules associated with proteins.[6] Either benzoxazolium-, benzothiazolium-, or benzoselenazolium-group was employed as an electron accepting group. In order to investigate the effect of hydrocarbon tail, the tail length was also varied.

The fluorescent stains were synthesized according to Scheme 1. Knoevenagel reactions of 4-(methyl-alkyl-amino)-benzaldehyde with 2,3-dimethylbenzoxazol-3-ium (**1**), 2,3-dimethylbenzothiazol-3-ium (**2**), or 2,3-dimethylbenzoselenazol-3-ium (**3**) were carried under refluxing condition in ethanol. Molecular structures of the fluorescent probes were confirmed by their spectroscopic data.[7]

As shown in Figure 1, the excitation maxima of the synthesized dyes are in the range of 500–550 nm. Both excitation and emission wavelengths increase in the order of **1**, **2**, and **3**. Spectra



Scheme 1. Structures and synthesis of fluorescent stains.

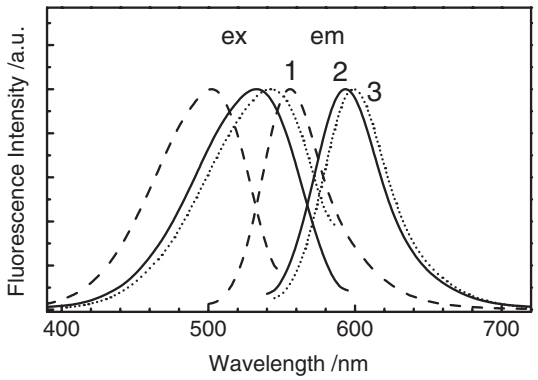


Figure 1. Normalized fluorescence excitation and emission spectra of the synthesized stains in ethanol. Dashed lines, solid lines and dotted lines represent **1d**, **2d**, and **3d**, respectively.

of benzooxazolium derivative (**1**) is about 50 nm blue-shifted compared to benzothiazolium (**2**) or benzoselenazolium derivatives (**3**). Nevertheless, all synthesized dyes can be excited efficiently by using commercially available light sources in gel scanners, such as 488 or 532-nm laser lights. Absorption and emission characteristics of the dyes in ethanol are summarized in Table 1.

The quantum yields of the dyes in aqueous solutions are exceptionally low, making the dyes virtually non-fluorescent.

Table 1. Fluorescence characteristics of the synthesized stains

Stain	λ_{abs} /nm	λ_{em} /nm	ϕ^a	Enhancement factor[b]
1d	500	555	0.037	110
2a	532	595	0.013	90
2b	531	596	0.019	40
2c	534	594	0.020	50
2d	534	596	0.024	170
2e	538	590	0.021	280
3d	544	599	0.023	366

Fluorescence spectra are measured with 1.0 μM dye solutions in ethanol. [a]Fluorescence quantum yields. [b]Fluorescence emission increase in the presence of 150 μg/mL BSA and 0.05% SDS compared to the one in the presence of 0.05% SDS only in aqueous 1.5% acetic acid solution.

However, the emission increased greatly in the presence of proteins exhibiting intense fluorescence in aqueous solutions. This drastic change of fluorescence spectra is demonstrated in Figure 2. It should be noted that this emission increase was observed only when both BSA and SDS are present, which indicates that the dyes interact with SDS molecules associated with proteins. The quantum yields of the dyes in BSA solution increased gradually with increasing BSA concentration, and then reached ≈0.1. The extent of enhancement increased in the order of **1d**, **2d**, and **3d** (Table 1). Also the length of hydrocarbon tail affected the emission increase. We found that this emission increase is largely attributed to the increase of fluorescence lifetime (inset of Figure 2). Binding of the dyes to BSA–SDS complex restricts mobility of the dyes and reduces nonradiative relaxation of the excited dye molecules, which in turn increases the fluorescence lifetime.

We attempted to visualize molecular weight marker proteins in SDS–PAGE by staining with the synthesized dyes. After the electrophoresis was completed, the gel was fixed followed by incubation in the staining solution containing 1 μM dye and 5% ethanol for 1 h. The resulting gel images (Figure 3) obtained by irradiating the gels with 532-nm light indicate that all synthesized dyes successfully stained proteins in SDS–PAGE with high

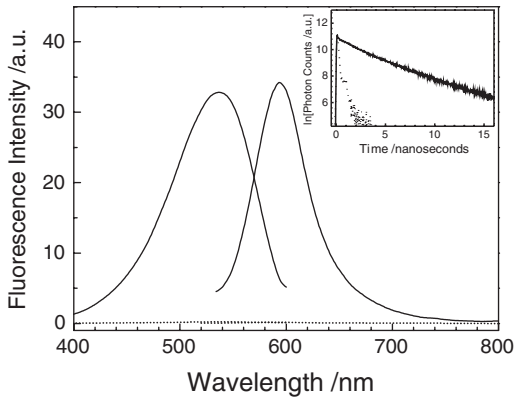


Figure 2. Fluorescence enhancement of **2a** in the presence of proteins. Solid lines represent excitation and emission fluorescence spectra in the presence of 150 μg/mL BSA and 0.05% SDS. Dotted lines are for the ones in the presence of 0.05% SDS only. Inset depicts the fluorescence lifetimes of **2a** in the presence ($\tau_{av} = 1.1$ ns, solid line) and absence of BSA ($\tau_{av} < 30$ ps, dotted line). All solutions contain 1.5% acetic acid. [**2a**] = 1 μM.

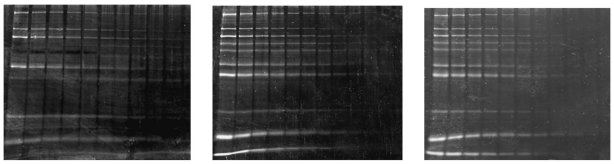


Figure 3. SDS–PAGE gel images stained with the synthesized dyes. Molecular weight marker proteins were loaded by twofold serial dilution starting from 100 ng/band at the most left lane.

sensitivity. Protein bands containing less than 1 ng proteins (8th lane) were readily visualized and the sensitivity was maintained after prolonged exposure to the laser light by scanning the gel repeatedly (>5 times attempted).

In conclusion, fluorescent dyes consisting of an environment sensitive fluorophore and a hydrocarbon tail can be excellent non-covalent protein stains for SDS–PAGE. Further effort for developing sensitive protein stains is underway in our group.

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7 Selected data for **1d**, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (t, 3H), 1.10–1.38 (m, 24H), 1.61 (t, 2H), 3.07 (s, 3H), 3.39 (t, 2H), 4.37 (s, 3H), 6.68 (d, 2H), 7.52 (d, 1H), 7.54 (d, 1H), 7.57 (d, 1H), 7.67 (t, 2H), 8.00 (d, 2H), 8.14 (d, 1H), HRMS (FAB^+) m/z calcd for $C_{32}H_{47}N_2O^+$, 475.3688 (M^+) observed, 475.3688; **2a**, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (t, 3H), 1.20–1.38 (m, 18H), 1.55 (t, 2H), 2.95 (s, 3H), 3.28 (t, 2H), 4.33 (s, 3H), 6.51 (d, 2H), 7.45 (td, 1H), 7.54–7.60 (m, 2H), 7.64 (d, 1H), 7.86 (d, 1H), 7.94 (d, 2H), 8.08 (d, 1H), HRMS (FAB^+) m/z calcd for $C_{29}H_{41}N_2S^+$, 449.2990 (M^+) observed, 449.2995; **2b**, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (t, 3H), 1.17–1.36 (m, 20H), 1.61 (t, 2H), 3.05 (s, 3H), 3.39 (t, 2H), 4.44 (s, 3H), 6.67 (d, 2H), 7.54 (td, 1H), 7.64 (td, 1H), 7.67 (d, 1H), 7.78 (d, 1H), 7.83 (d, 1H), 7.95–8.05 (m, 3H), HRMS (FAB^+) m/z calcd for $C_{30}H_{43}N_2S^+$, 463.3147 (M^+) observed, 463.3159; **2c**, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (t, 3H), 1.16–1.37 (m, 22H), 1.59 (t, 2H), 3.02 (s, 3H), 3.56 (t, 2H), 4.40 (s, 3H), 6.63 (d, 2H), 7.53 (td, 1H), 7.61 (td, 1H), 7.64 (d, 1H), 7.75 (d, 1H), 7.85 (d, 1H), 7.98 (d, 2H), 8.00 (d, 1H), HRMS (FAB^+) m/z calcd for $C_{31}H_{45}N_2S^+$, 477.3303 (M^+) observed, 477.3306; **2d**, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (t, 3H), 1.21–1.35 (m, 26H), 1.61 (t, 2H), 3.01 (s, 3H), 3.38 (t, 2H), 4.43 (s, 3H), 6.66 (d, 2H), 7.55 (td, 1H), 7.64 (td, 1H), 7.67 (d, 1H), 7.78 (d, 1H), 7.83 (d, 1H), 7.92–8.00 (m, 3H), HRMS (FAB^+) m/z calcd for $C_{32}H_{47}N_2S^+$, 491.3460 (M^+) observed, 491.3447; **2e**, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (t, 3H), 1.18–1.35 (m, 30H), 1.59 (t, 2H), 3.03 (s, 3H), 3.36 (t, 2H), 4.41 (s, 3H), 6.64 (d, 2H), 7.53 (td, 1H), 7.63 (td, 1H), 7.65 (d, 1H), 7.75 (d, 1H), 7.84 (d, 1H), 7.96 (d, 2H), 8.00 (d, 1H), HRMS (FAB^+) m/z calcd for $C_{35}H_{53}N_2S^+$, 533.3929 (M^+) observed, 533.3914; **3d**, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (t, 3H), 1.12–1.38 (m, 22H), 1.61 (t, 2H), 2.02 (t, 2H), 3.03 (s, 3H), 3.36 (t, 2H), 4.26 (s, 3H), 6.60 (d, 2H), 7.42 (td, 1H), 7.49–7.55 (m, 2H), 7.59 (d, 1H), 7.70 (d, 1H), 7.87 (d, 2H), 8.30 (d, 1H), HRMS (FAB^+) m/z calcd for $C_{32}H_{47}N_2Se^+$, 539.2904 (M^+) observed, 539.2921.

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