Red Luminescent Squarylium Dyes for Noncovalent HSA Labeling

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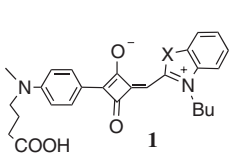
Asymmetric squarylium fluorophores with 618–680 nm emissions exhibit significantly enhanced fluorescence and increased quantum yield (maximum 97%) by the formation of 1:1 dye–protein complexes when bound as non-covalent labeling probes to human serum albumin (HSA).

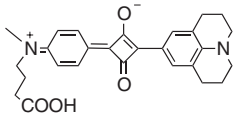
Fluorescent probes for protein labeling have widely been used in clinic and analytical applications.[1] The ideal probe for protein labeling is devoid of its own fluorescence in the absence of protein; has a high binding affinity for proteins; and shows enhanced fluorescence after binding to proteins only. Though most commercially available fluorescent probes are covalent labeling probes for proteins, noncovalent labeling offers a simple and faster labeling procedure than required by covalent labeling.[2] Long-wavelength absorbing and emitting fluorophores based on cyanine, Rhodamine, porphyrin, and phthalocyanine dyes represent typical fluorescent probes for protein.[3]

Squarylium dyes have a zwitterion structure due to the central squarate bridge to exhibit effective light absorption and have found use in applications such as photoconductors in copy devices,[4] organic solar cells,[5] and optical recording media.[6] However, there is little information on their fluorescence properties. Some squarylium dyes used as noncovalent protein probes have resulted in enhanced fluorescence and increased quantum yield when bound to bovine serum albumin (BSA).[7–9] However, the quantum yield of the squarylium dye–protein complex remains at 78%.[7] Additionally, Oswald et al. showed that two reactive squarylium dyes for covalent attachment to amino group of proteins resulted in enhanced fluorescence lifetime and increased quantum yields.[10]



1

X
a: S
b: O
c: $C(CH_3)_2$
d: -CH=CH-



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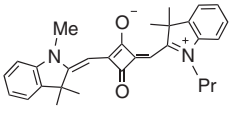

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In the present work, we prepared new asymmetric squarylium dyes, which displayed greatly improved fluorescence intensities and quantum yields in a trizma buffer containing human serum albumin (HSA). These are presented as new noncovalent HSA labeling agents. They can be dissolved first in methanol followed by dilution with aqueous trizma buffer without precipitation.

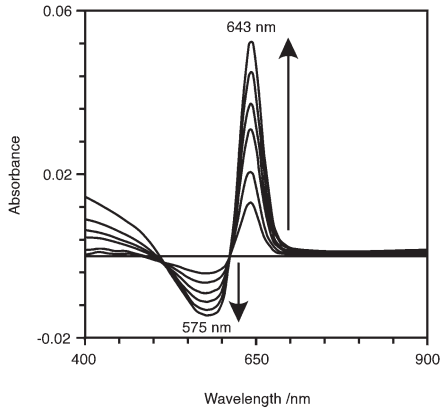

Figure 1. Difference absorbance spectra for the titration of 5.0×10^{-7} M dye **1c** with increasing amounts of HSA in aqueous trizma buffer (pH 7.4) at 298 K.

The squarylium dyes **1** and **2** were prepared from various heterocyclic cations including an active methyl group or julolidinyl derivative and 3-(*N*-[3-carboxylpropyl]-*N*-methylamino)-phenyl-4-hydroxycyclobut-3-ene-1,2-dione by the method described previously.[11] Characterizations of these dyes as fluorescent probes are summarized in Table 1.

The absorbance spectra of squarylium dye **1** showed a sharp band at 566–615 nm and their molar absorptivities (ε) were between 86000 and 190000 $M^{-1}cm^{-1}$ in methanol. Their maxima were broadened and significantly blue-shifted by 11–54 nm, and their ε were decreased in aqueous trizma buffer (adjusted to pH 7.4), compared with those in methanol. This large blue-shift suggests that the squarylium dyes may form aggregates in aqueous trizma buffer. Figure 1 shows the difference spectra for the titration of dye **1c** with HSA in trizma buffer. A new peak at

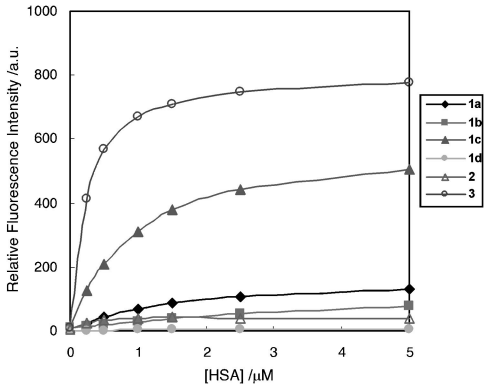

Figure 2. Fluorescence intensities at λ_f of 5.0×10^{-7} M dyes **1**–**3** as a function of the HSA concentration in trizma buffer (pH 7.4) at 298 K.

Table 1. Absorption maxima (λ_{abs}), molar absorptivity (ε), fluorescence maxima (λ_f), and quantum yield (Q_f) of unsymmetrical squarylium dyes **1–3** in the absence and in the presence of HSA

Dye	Without HSA[a]				With HSA[b]			
	λ_{abs}/nm	$\triangle\lambda$[c]/nm	log ε	Q_f[d]	λ_{abs}/nm	λ_f/nm	Q_f[d]	K_s/M^{-1}
1a	564	−35	4.81	0.05	621	656	0.70	8.7×10^5
1b	550	−16	4.89	0.05	550	618	0.39	1.1×10^6
1c	604	−11	4.96	0.03	643	654	0.92	8.0×10^5
1d	558	−54	4.66	0.04	656	677	0.06	1.7×10^5
2	658	+8	5.08	0.01	658	680	0.34	5.8×10^6
3	622	−5	5.11	0.02	640	645	0.97	3.8×10^6

[a] 5.0×10^{-7} M solution of dye dissolved in trizma buffer (pH 7.4) at 298 K. [b] 5.0×10^{-7} M solution of dye dissolved in trizma buffer (pH 7.4) with 5.0×10^{-6} M HSA at 298 K. [c] $\triangle\lambda = \lambda_{\text{trizma buffer}} - \lambda_{\text{MeOH}}$. [d] The Q_f was determined relative to that of Rhodamine B (0.97). Excitations at 630 nm, 550 nm, 630 nm, 630 nm, 658 nm and 630 nm for **1a**, **1b**, **1c**, **1d**, **2**, and **3**, respectively. The λ_f in the absence of HSA was same to that in the presence of HSA.

643 nm with an isosbestic point at 604 nm appeared on addition of protein HSA to the dye, which was assigned to the dye–HSA complex. This λ_{max} was shifted bathochromically by 39 nm relative to the λ_{max} in trizma buffer without HSA. Similar shifts were observed for **1a**, **1d**, and **3**, while λ_{max} for **1b** and **2** did not shift upon addition of HSA. The absorbance maximum for the complex between 621 and 658 nm allows for the use of commercially available diode lasers, and the large shift of λ_{max} accompanying the formation of the dye–protein complex is favorable for high sensitivity in analytical applications.

The fluorescence intensities recorded for the titration of dyes **1–3** with HSA in trizma buffer are shown in Figure 2. Titration of dyes **1–3** (5.0×10^{-7} M) with varying concentrations of HSA (ranging from 0 to 50.0×10^{-7} M) in trizma buffer led to significant enhancements in fluorescence. The most significant enhancement was observed upon the addition of HSA to **1c** and **3**. A limit of detection (S/N = ca. 15) of 2.0 nM HSA labeled with **1c** was observed by means of a conventional fluorescence spectrometer. The maxima in the resulting Job's plots to determine the predominant stoichiometries of the HSA:dye complexes occurred at an HSA mole fraction of 0.5 in the trizma buffer for HSA with **1c**, which corresponds to a 1:1 protein–dye stoichiometry. The Job's plot for HSA with **1a**, **b**, **d**, **2**, and **3** similarly predicted a predominant stoichiometry of 1:1.

The stability constant K_s of the HSA–dye complex formed with a 1:1 stoichiometry was determined according to the method reported by Tarazi and co-workers.[12] Stability constants on the order of ca. 10^6 M^{-1} for dyes **1–3**, except for **1d**, were observed (Table 1), indicating that strong interactions with HSA are characteristic of asymmetric squarylium dyes. It is well known that HSA has three sites; called digitoxin, warfarin, and diazepam sites,[13] for interactions with drugs. Though warfarin has a coumarin fluorophore, the fluorescence quantum yield of warfarin–HSA complex in trizma buffer was low (0.11). However, titration of **1c** to the warfarin–HSA complex led to significant enhancement in fluorescence at 654 nm and decrease at 380 nm indicating that the dye was binding at the warfarin site. As shown in Table 1, fluorescence maxima of **1–3** were located at 618–680 nm in the aqueous trizma buffer. These dyes exhibit very low fluorescence quantum yields in the absence of HSA, but these yields are significantly increased when bound to HSA. Specifically, dyes **1c** and **3** exhibit the largest fluorescence quantum yields (0.92 and 0.97) in the presence of HSA. These results suggest that dyes **1–3** can serve as excellent noncovalent labeling agents for HSA. Furthermore, the fluorescence lifetimes of **1a–1c** are 1.57, 2.09, and 2.32 ns, respectively. However, these lifetimes increased substantially to 2.86, 2.54, and 3.21 ns, respectively, upon binding to HSA.

The largest increase in fluorescence quantum yield was displayed by dye **1** when bound to HSA, and this clearly offers an advantage in analytical and clinical chemistry applications. The fact that squarylium dyes **1c** and **3**, each containing an indolenium ring, display an increase in fluorescence quantum yield upon binding to HSA can be especially advantageous in such applications, because the emission was predominantly due to the HSA-bound species, with little emission from the free dye.

The fluorescence emissions of dye **1a** have been also shown to be enhanced upon complexation with proteins, such as BSA, β-lactoglobulin A and trypsinogen.[14] The great enhancement in fluorescence for BSA, as well as HSA, and poor fluorescence enhancements for β-lactoglobulin A and trypsinogen were observed, indicating that dye **1a** will show high selectivity between HSA and β-lactoglobulin A (or trypsinogen).

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