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Synthesis, Spectral Properties, and Detection Limits of Reactive Squaraine Dyes, a New Class of Diode Laser Compatible Fluorescent Protein Labels

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We describe the synthesis and spectral characterization of two reactive long-wavelength fluorescence labels (Sq635-m and Sq635-b), having either one or two *N*-hydroxysuccinimidyl esters. Both are squaraine derivatives and consist of a cyanine-type chromophore and a central squarate bridge. To improve water solubility, we introduced two sulfonic acid groups into the heterocyclic ring systems, and for covalent attachment to proteins, a reactive *N*-hydroxy-succinimide ester (NHS ester) was synthesized. The squaraine markers exhibit low quantum yields in water ($\phi = 0.15$) and high quantum yields ($\phi = 0.6-0.7$) when bound to proteins. The absorption maxima at 635 nm in water and at approximately 645 nm when bound to proteins allow excitation with commercially available diode lasers. The detection limit of a representative squaraine dye in blood was estimated to be half that of a commonly used fluorophore.

INTRODUCTION

Fluorescence detection is widely used in immunoassays, hybridization assays, enzymatic reactions and other analytical procedures (*1*−*3*). The advantages of fluorescence detection at long-wavelength excitation are the decreased autofluorescence from cells and tissues and the use of inexpensive laser light sources such as diode lasers operating at 635, 645, and 650 nm. It is well-known that the autofluorescence of biological samples decreases with increasing wavelength, particularly beyond 600 nm. Only a few fluorescent probes exist which absorb in the red or near-infrared (NIR) region and even fewer of them are available in a reactive form (*4*−*8*). Amine-reactive functionalities such as isothiocyanate and *N*-hydroxysuccinimide esters and thiol-reactive iodoacetamide and maleimide groups can be used to covalently attach marker molecules to drugs, DNA, or antibodies (*5*, *6*).

The synthesis and spectral characterization of a squaraine derivative for covalent attachment to biomolecules was already reported (*4*). Squaraines, which are 1,3-disubstituted squaric acid derivatives, show high-absorption coefficients ($\epsilon > 200.000$ L mol^{-1} cm^{-1}) in the red region, display a high photostability, and allow the introduction of reactive functional groups such as NHS[1] esters. These dyes exhibit good quantum yields on binding to proteins but insufficient water solubility. The protein conjugates precipitate, in particular at higher dye-to-protein ratios (*4*, *9*). The water solubility of dyes can be improved by introducing sulfonic acid groups (*4*).

Reactive squaraines developed by Mank et al. were used as precolumn derivatization reagents for trace analysis of small molecules (e.g., drugs) in combination with diode laser induced fluorescence and allowed detection of analyte concentrations as low as 50−100 nmol L^{-1} (*6*). We now introduce the second generation of squaraine dyes with improved spectral and chemical properties. Specifically, we report the synthesis of a symmetrical, "bis-reactive" (Sq635-b), and an unsymmetrical, "monoreactive" (Sq635-m), water-soluble, squaraine-dye, and their conjugation to BSA, HSA, and anti-HSA. We also discuss their absorption and fluorescence spectral properties and compare the detection limit of one representative dye to CY5 in blood.

EXPERIMENTAL PROCEDURES

Chemicals, Proteins, and Media. Squaric acid, squaric acid dibutyl ester, and 6-bromohexanoic acid were

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[1] Abbreviations: BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; *D*/*P*, dye-to-protein ratio; HSA, human serum albumin; hCG, human chorionic gonadotropin; mp, melting point; NHS, *N*-hydroxysuccinimide; Sq635-b, 2,4-bis[*N*−(ε-butoxycarbonylpentanyl)-3,3-dimethyl-5-sulfo-2-indolinylidenemethyl]cyclobutenediylium-1,3-diolate; Sq635-m, 2-[*N*-(ε-butoxycarbonylpentanyl)-3,3-dimethyl-5-sulfo-2-indolinylidenemethyl]-4-[3,3-dimethyl-5-sulfo-2-indolinylidenemethyl] cyclobutenediylium-1,3-diolate; TMS, tetramethylsilane; TSTU, *N*,*N*,*N*,*N*-tetramethyl(succinimido)uronium tetrafluoroborate.

purchased from Aldrich. Cy5 was obtained from Amersham Life Science, Pittsburgh. BSA was purchased from Serva Feinchemica, Heidelberg. HSA and anti-HSA were obtained from Sigma. All other chemicals and solvents were obtained from Merck. All chemicals were used as received. Phosphate buffer of pH 7.3, 0.1 M, 3.17 g of NaH_2PO_4 and 13.7 g of Na_2HPO_4, was dissolved in 1 L of doubly distilled water. Bicarbonate buffer of pH 9, 0.05 M, 2.1 g of $NaHCO_3$, was dissolved in 500 mL of doubly distilled water.

Purification and Spectroscopic Analysis. Analytical and preparative thin-layer chromatography was carried out on RP-18 F_{254} silica plates from Merck. Ion-exchange chromatography was performed using a weakly acidic cation exchanger Amberlite IRC-50. Gel permeation chromatography was carried out using Sephadex G50 as the stationary phase (0.5×20 cm column) and a 100 mM phosphate buffer solution (pH 7.3) as eluent. Melting points were measured with apparatus SMP-20 from Büchi and are not corrected. pH values were measured at room temperature with a pH meter WTW 538.

UV spectra were recorded with a U-3000 spectrophotometer (Hitachi). Emission spectra were acquired with a luminescence spectrometer from Aminco (Series 2, from SLM) with a standard 150 W xenon lamp as the excitation source. ^1H NMR spectra were recorded with a 250 MHz PFT-NMR spectrometer (AC 250, from Bruker). The internal standard was TMS. The chemical shifts are given in parts per million. The following abbreviations were used to describe the signals: s = singlet, d = doublet, dd = double of doubletts, t = triplet, m = multiplet. Mass spectral analysis was performed at the Central Analytical Department at the University of Regensburg, Germany.

The BSA concentration was determined using the BCA Protein Assay Reagent Kit from Pierce (Rockford, IL). Quantum yields were determined relative to Cy5 assuming a quantum yield of 0.25 for $\lambda_{exc} = 650$ nm for Cy5 using the formula $\Phi = \Phi_{Cy5}(F/F_{cy5})$, where F is $\int I(\lambda) \, d\lambda$.

Syntheses. Synthesis of *p*-hydrazinobenzenesulfonic acid (*10*), 2,3,3-trimethylindole-5-sulfonic acid potassium salt (*4*, *5*, *11*), and 1-(ϵ-carboxypentanyl)-2,3,3-trimethylindolenium-5-sulfonic acid potassium salt (**1**) (*5*) was carried out using literature procedures.

Synthesis of Sq635-b-butylester (2) (*4*). To 1 g (2.17 mmol) of 1-(ϵ-carboxypentanyl)-2,3,3-trimethylindolenium-5-sulfonic acid potassium salt (**1**) was added 120 mg (1.03 mmol) of squaric acid, and the mixture was refluxed in 50 mL of a 1-butanol:toluene (1:1, v/v) mixture for 22 h using a Dean–Stark trap filled with 4A molecular sieve. After cooling, the solvent was removed, and the product purified by preparative thin-layer chromatography using RP-18 glass plates and methanol:water (2:1, v/v) as eluent to give 90 mg (22%) of **2**. R_f: 0.47 (RP-C18, methanol:water 2:1); mp >300 °C: FAB-MS, *m/e* (M⁺, dianion) for $C_{46}H_{58}N_2O_{12}S_2K_2$, calcd 895.1, found 894.8. ^1H NMR (D_2O): δ 7.7−7.1 (m, 6H), 5.7 (s, 2H), 3.7 (t, 4H, $J = 6.5$), 2.0 (t, 4H, $J = 7$ Hz), 1.55−0.9 (m, 24H), 1.45 (s, 12H), 0.5 (t, 6H, $J = 7$ Hz).

Synthesis of Sq635-b-acid (3). To 50 mg (0.05 mmol) of Sq635-b-butylester (**2**) was added 1 mL of water and 20 mL of 1 M HCl. The mixture is heated to 100 °C for 80 min. At the end of the reaction, 5 mL of 1 mol/L HCl was added. After cooling, the solvent was removed and the product vacuum dried. The product was used without further purification (43 mg, 99%). R_f: 0.75 (RP-C18, methanol:water 2:1), mp >300 °C, FAB-MS, *m/e* (M⁺, dianion) for $C_{38}H_{42}N_2O_{12}S_2K_2$, calcd 782.9, found 783.0.
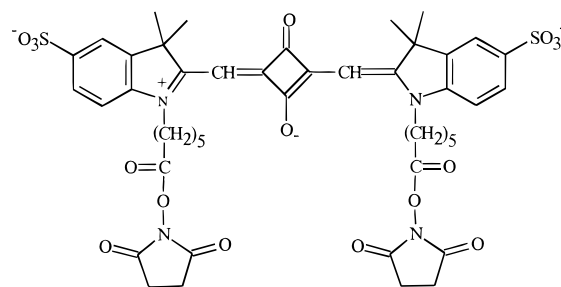
Sq635-b-NHS-ester

λ_{max}(abs.): 634 nm, λ_{max}(em.): 648 nm

$\epsilon = 140,000$ [l mol⁻¹ cm⁻¹]
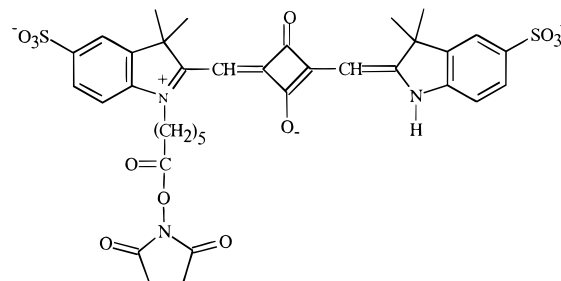
Sq635-m-NHS-ester

λ_{max}(abs.): 634 nm, λ_{max}(em.): 646 nm

$\epsilon = 120,000$ [l mol⁻¹ cm⁻¹]

Figure 1. Chemical structure of the Sq635-b- and Sq635-m-NHS ester.

Cy5
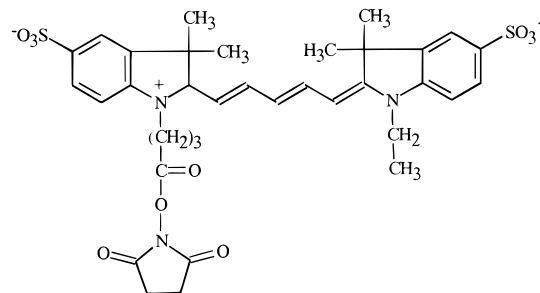
λ_{max}(abs.): 647 nm, λ_{max}(em.): 664 nm

$\epsilon = 250,000$ [l mol⁻¹ cm⁻¹]

Figure 2. Chemical structure of the Cy5-NHS ester.

^1H NMR (D_2O): δ 7.8−7.3 (m, 6H), 5.9 (s, 2H), 4.2 (t, 4H, $J = 6.5$ Hz), 2.4 (t, 4H, $J = 7$ Hz), 1.95−1.3 (m, 12H), 1.77 (s, 12H).

Synthesis of Sq635-b-NHS-ester (4) (a) with TSTU [N,N,N′,N′-tetramethyl(succinimido)uronium tetrafluoroborate] (12). To 43 mg (0.05 mmol) of Sq635-b-acid (**3**) in 1 mL of DMF, 1 mL of dioxane, and 0.5 mL of water were added 26 μL (0.15 mmol) of diisopropylethylamine and 38 mg (0.126 mmol) of TSTU. After 30 min, the mixture was filtered, and the solvents were removed in a vacuum. The product was dried over P_2O_5 and used without further purification. Yield: 40 mg (76%). R_f: 0.82 (RP-C18, methanol:water 2:1); mp >300 °C; FAB-MS, *m/e* (M⁺, dianion) for $C_{46}H_{48}N_4O_{16}S_2K_2$, calcd 977.0, found 977.1. ^1H NMR (d_6-DMSO): δ 7.65−7.2 (m, 6H), 5.8 (s, 2H), 4.2 (t, 4H, $J = 6.5$ Hz), 2.9−2.6 (m, 12H), 1.65−1.4 (m, 12H), 1.75 (s, 12H).

n-butanol/toluene

(1)

(2)

HCl

(3)

a)

TSTU
DMF
H$_2$O
1,4-dioxane

b)

NHS
DCC
NEt$_3$

DMF

Sq635—C—NH—| Protein |

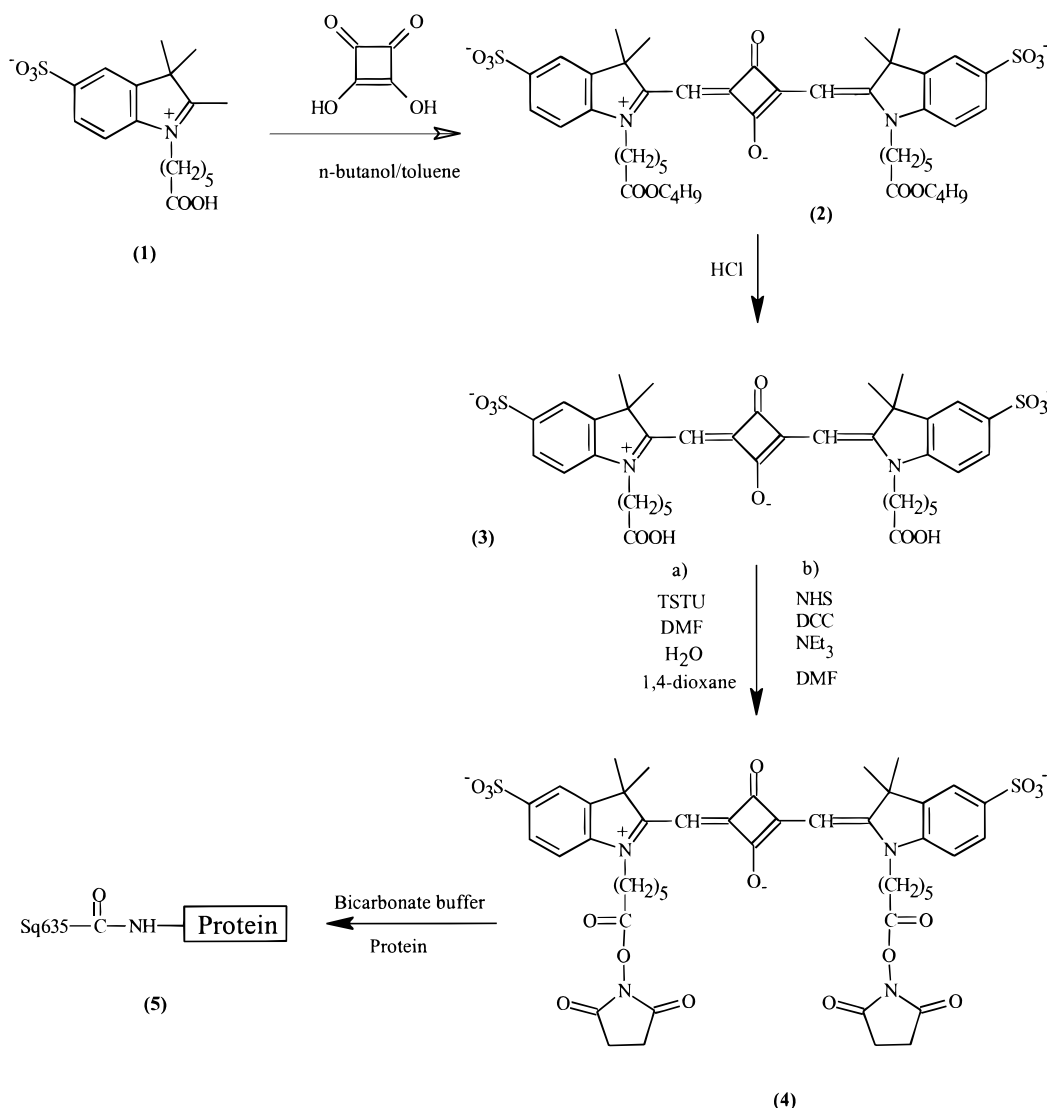Bicarbonate buffer

Protein

(5)

(4)

Figure 3. Synthetic pathway for the bis-functional squaraine-NHS ester.

Synthesis of Sq635-b-NHS-ester (4) (b) with NHS/ DCC. A total of 14 mg (0.069 mmol) of dicyclohexylcarbodiimide (DCC) and 8 mg (0.069 mmol) of N-hydroxysuccinimide (NHS) was added to 20 mg (0.023 mmol) of Sq635-b-acid **(3)** in 1 mL of anhydrous DMF. The solution was stirred for 24 h at room temperature and filtered. The solvent was removed under reduced pressure, and the remaining solid was triturated with ether and dried over P$_2$O$_5$ (22 mg, 91%). R_f: 0.82 (RP-C18, methanol: water 2:1); mp >300 °C; FAB-MS, m/e (M$^+$, dianion) for C$_{46}$H$_{48}$N$_4$O$_{16}$S$_2$K$_2$, calcd 977.0, found 977.2. ^{1}H NMR (d_6-DMSO): δ 7.65−7.2 (m, 6H), 5.8 (s, 2H), 4.2 (t, 4H, $J =$ 6.5 Hz), 2.9−2.6 (m, 12H), 1.65−1.4 (m, 12H), 1.75 (s, 12H).

Synthesis of Sq635-m-acid (8). To 47 mg (0.1 mmol) of 1-(ε-carboxypentanyl)-2,3,3-trimethylindolenium-5-sulfonic acid potassium salt **(1)** was added 22 μL (0.1 mmol) of squaric acid dibutyl ester, and the mixture was refluxed in 8 mL of ethanol with 140 μL of triethylamine for 30 min. Subsequemtly, 220 μL of 1 M aqueous NaOH was added, and the mixture was refluxed for 30 min. After cooling to room temperature, 2.3 mL of 1 M hydrochloric acid was added, and the solvent was removed under reduced pressure to obtain the monosubstituted squaraine derivative **(7)**.

The residue was refluxed with 24 mg (0.09 mmol) of 2,3,3-trimethylindole-5-sulfonic acid potassium salt in a butanol:toluene mixture (1:1 v/v) for 1 h. Water was removed as an azeotrope using a Dean−Stark trap. After cooling, the solvents were removed using a rotary evaporator. The product was treated with 100 μL of methanol, collected under reduced pressure, and purified on preparative TLC (RP-18 F$_{254S}$) using methanol:water (2:1 v/v) as the eluent. Yield: 31 mg (33%). R_f: 0.50 (RP-C18, methanol:water 2:1); mp >300 °C. FAB-MS, m/e (M$^+$, dianion) for C$_{32}$H$_{32}$N$_2$O$_{10}$S$_2$K$_2$, calcd 668.1, found 668.5. ^{1}H NMR (D$_2$O): δ 7.75−7.5 (m, 4H), 7.15−6.95 (m, 2H), 5.55 (s, 1H), 5.35 (s, 1H), 4.55 (t, 2H, $J =$ 6.5 Hz), 2.05−2.3 (m, 2H), 1.5−1.2 (m, 6H), 1.25 (t, 12H).

The activation to the NHS-ester was carried out analogous to Sq635-b-acid **(3)** using procedure b. R_f: 0.55 (RP-C18, methanol:water 2:1), mp >300 °C. FAB-MS, m/e (M$^+$, dianion) for C$_{36}$H$_{35}$N$_3$O$_{12}$S$_2$K$_2$, calcd 766.1, found 766.4. ^{1}H NMR (D$_2$O): δ 7.85−7.5 (m, 4H), 7.15−6.9 (m, 2H), 5.55 (s, 1H), 5.35 (s, 1H), 4.45 (t, 2H, $J =$ 6.5 Hz), 2.7 (s, 4H) 2.05−2.35 (m, 2H), 1.5−1.2 (m, 6H), 1.25 (t, 12H).

General Protein Labeling Procedures and Determination of Dye-to-Protein Ratios. Protein labeling reactions were carried out using 50 mM bicarbonate buffer (pH 9.0). A stock solution of 1 mg of dye in 100 μL of anhydrous DMF was prepared; 10 mg of protein was dissolved in 1 mL of a 100 mM bicarbonate buffer, pH 9.1, and various amounts of dye from the stock solution
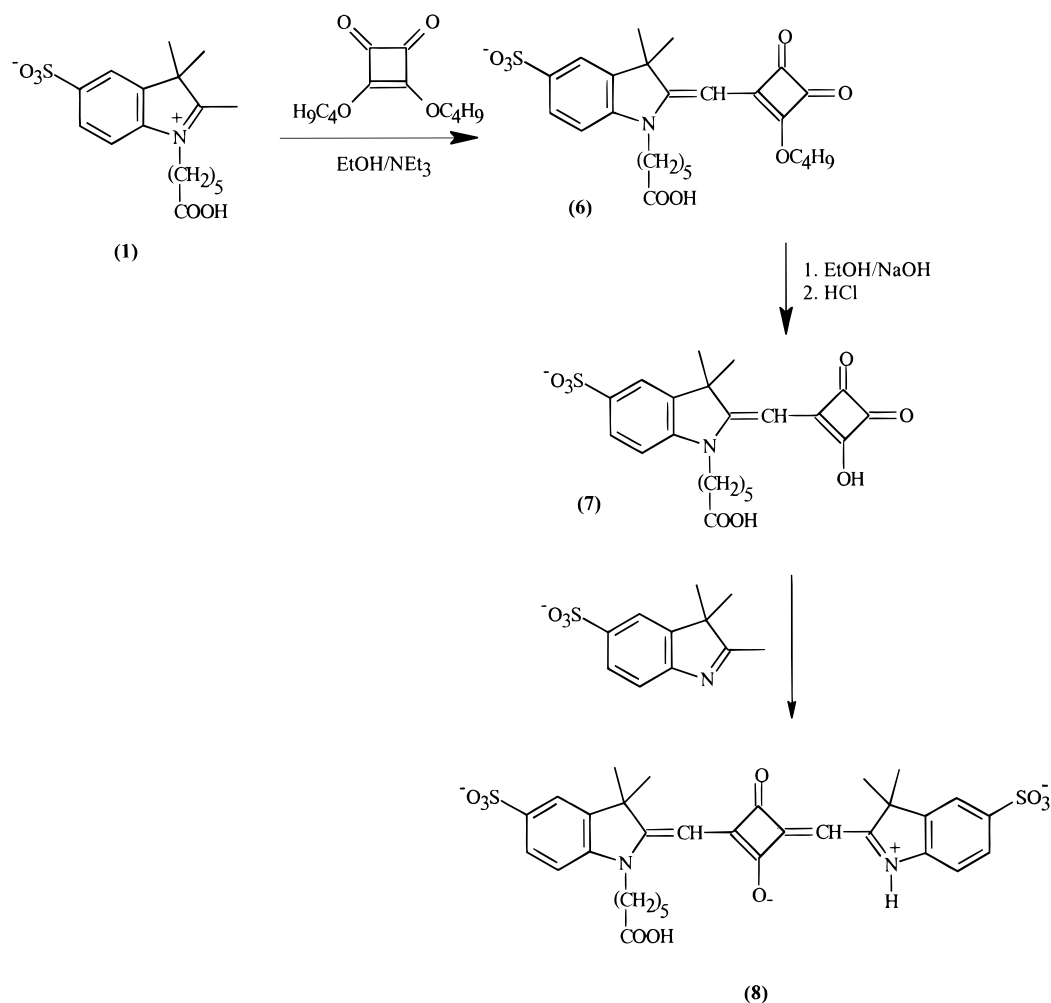
Figure 4. Synthetic pathway for the monofunctional squaraine-NHS ester.

were added, and the mixture was allowed to stir for 24 h at room temperature.

Unconjugated dye was separated from the labeled proteins using gel permeation chromatography with Sephadex G50 (0.5 cm × 20 cm column) and a 22 mM phosphate buffer solution of pH 7.3 as the eluent. The first colored band contained the dye–protein conjugate, while the blue band with a much higher retention time contained the separated free dye. A series of labeling reactions as described above was set up to obtain different dye-to-protein ratios. Compared to the free forms, the protein-bound forms of the dyes show distinct changes in their spectral properties.

The molar dye-to-protein ratio (degree of labeling) for each dye conjugate was calculated as the molarity of dye divided by the molarity of protein. The dye concentration of the conjugate was determined according to Lambert–Beer's Law from the absorbance of the dye at the absorption maximum. The protein concentration was determined using the BCA Protein Assay Reagent Kit from Pierce (Rockford, IL). The dye-to-protein ratio (D/P) gives the number of dye molecules covalently bound to the protein.

Determination of the Extinction Coefficients. Sq635-b-butylester (**2**) was purified on preparative TLC. The K^+ ions were replaced by H^+ ions by ion-exchange chromatography using Amberlite IRC-50 as the stationary phase and methanol as the eluent. Sq635-b-butylester (**2**) (0.55 mg) was dissolved in 100 mL of doubly distilled water. From this stock solution, three dilutions (1:20,

1:50, and 1:100) were made, and the absorbances were measured. The extinction coefficient was calculated according to Lambert–Beer's law and was found to be 140 000 L/(mol cm) for Sq635-b-butylester (**2**). In an analogous way the extinction coefficient for Sq635-m-acid (**8**) was determined to be 120 000 L/(mol cm). Both values refer to 634 nm.

Determination of the Detection Limits in Blood. Human blood was diluted 1:20 with phosphate buffer solution (pH 7.2). The absorbances of 50 nmol/L solutions of Sq635-b-NHS-ester (**4**), Sq635-b-anti-HSA, Cy5-NHS-ester, and Cy5-HSA were measured, and the concentrations were determined via Lambert–Beer's law assuming an extinction coefficient of 140 000 L/(mol cm) for Sq635-b-NHS-ester (**4**) and 250 000 L/(mol cm) for Cy5-NHS-ester. The emission spectra (Figure 6) of the solutions were measured by exciting at 600 nm using a standard xenon lamp (150 W) as the excitation source. The dye-to-protein ratios for Sq635-b-anti-HSA and Cy5-HSA are 0.49 and 1.1, respectively.

RESULTS

The synthetic pathway and the structures of the squaraine dyes are shown in Figures 1–4. While the synthesis of the symmetrical Sq635-b-acid (**3**) was achieved in a direct procedure (Figure 3), the synthesis of the unsymmetrical dye required the synthesis of a monosubstituted key intermediate (**6**) (Figure 4) that was hydrolyzed to the intermediate (**7**) and subsequently reacted
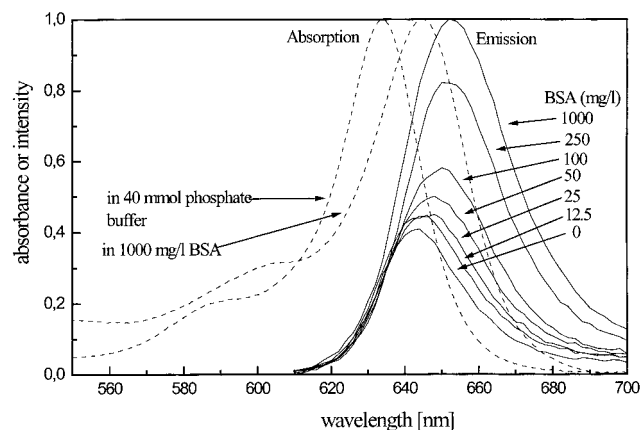
Figure 5. Absorption and relative emission spectra of Sq635-b-butylester in buffer and at various concentrations of BSA (λ_{ex} = 600 nm).
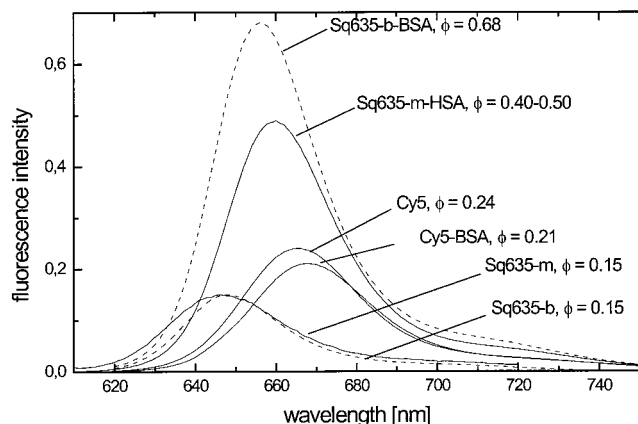

Figure 6. Relative emission spectra of Sq635-b, Sq635-m, and Cy5, free (NHS-ester) and conjugated to protein in buffer. The excitation wavelength was 600 nm using a standard 150 W xenon lamp.

with a second nonidentical methylene base to give the unsymmetrical Sq635-m-acid (**8**).

Two approaches were used to evaluate the potential of the squaraine dyes as protein labels. In the first, a nonreactive dye was mixed with increasing amounts of BSA. The extent of noncovalent binding of dyes to BSA was estimated by an increase of the relative fluorescence intensity (Figure 5). In the second approach, a reactive derivative of the dye was used for a covalent attachment procedure. In this case, the unlabeled dye was removed from the dye conjuagate using standard methods such as size-exclusion chromatography or dialysis.

The absorption and emission spectra of Sq635-b-butylester (**2**) at various concentrations of BSA are shown in Figure 5. The absorption maximum of the Sq635-b-butylester (**2**) at 635 nm shifts to 645 nm upon noncovalent binding to BSA, which makes the dye suitable for excitation with a 635 or 645 nm diode laser source. Compared to water, the emission maximum of (**2**) exhibits a small bathochromic shift of 14 nm on noncovalent binding to BSA, and the fluorescence intensity increases about two and a half times on addition of 1000 mg/L BSA. This suggests that sulfonated squaraines are suitable as fluorescent labels and would display good quantum yields when covalently bound to proteins.

Figure 6 shows a comparison of the relative emission spectra of Sq635-b, Sq635-m, and Cy5 both in free (NHS-ester) and protein-conjugated form in phosphate buffer of pH 7.3. The emission maximum of free Sq635-b-NHS-
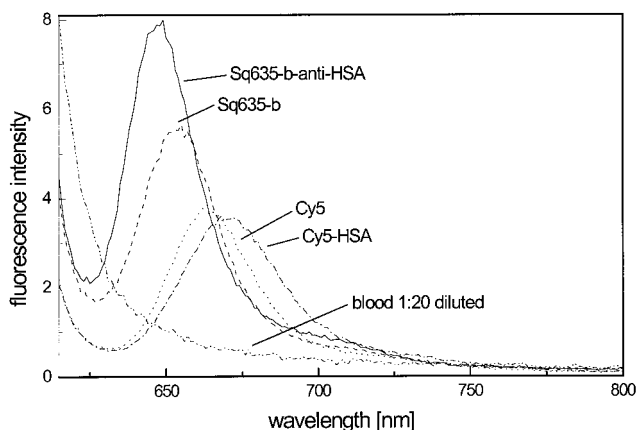
ester (**4**) at 648 nm shifts to 656 nm when covalently bound to protein. Similarly, the emission maximum of free Sq635-m-NHS-ester (**9**) at 646 nm shifts to 660 nm when bound to protein. The emission maximum of free Cy5-NHS-ester at 664 nm shifts to 668 nm when covalently linked to BSA. Importantly, the quantum yield of Sq635-b-NHS-ester (**4**) and of the Sq635-m-NHS-ester (**9**) increases on covalent attachment to protein from 0.15 to 0.68 and from 0.15 to 0.50, respectively. In contrast to the squaraine dyes, Cy5 shows a decrease in its quantum yield when bound to proteins, which lowers the ultimate sensitiviy of an assay using such dye conjugates.

In Figure 7, the detection limits of free and anti-HSA labeled Sq635-b-NHS-ester (**4**) are compared to those of free and HSA-labeled Cy5-NHS-ester. The concentrations of the dyes are 50 nmol/L. At 600 nm, the Cy5-NHS-ester and the Sq635-b-NHS-ester (**4**) have similar extinction coefficients. This wavelength was used for excitation of the blood samples. The data show that the detection limit of the squaraine dye can be as much as twice that of Cy5 due to the higher quantum yields of (**4**).

Lifetime-based sensing applications (*13–15*) using long-wavelength probes have gained increased attention in the last couple of years since convenient and inexpensive light sources and detection systems are available in the wavelength range beyond 600 nm. There are two widely used methods for the measurement of fluorescent lifetimes: the time domain and the frequency domain method. The frequency domain method uses sinusoidally modulated light for excitation of a fluorophore, and the phase shift (phase angle) and demodulation (modulation) of the emission (Figure 8), relative to the incident light, is used to calculate the lifetime or decay time (*16*).

Table 1 summarizes the intensity decays for the Sq635-b-butylester (**2**), Sq635-b-NHS-ester (**4**), Sq635-b-BSA, and Cy5 as presented in Figure 8. The free dyes (**2** and **4**) display two similar decay times. The mean lifetime of the NHS-ester **2** appears to be longer which is due to the higher fractional intensity of its longer component (Table 1).

On binding to BSA, the mean lifetime of the Sq635-b-NHS-ester (**4**) increased more than 4-fold due to the long decay time of 2.81 ns of the major component with a fractional intensity of 0.76. In addition, the intensity of the BSA-bound form became more heterogeneous. The increase of the mean lifetime from 0.43 to 2.26 ns is in good agreement with the 4-fold increase in the quantum yield for the free and BSA-bound forms of the squaraine
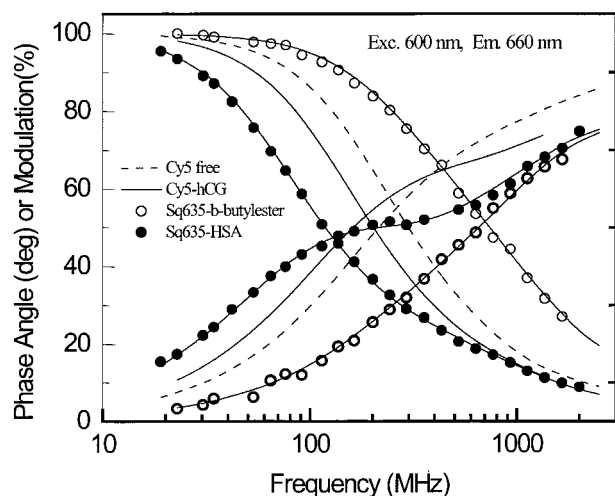
Figure 8. Frequency responses (phase angle and modulation) of Sq635-b-butylester, Sq635-b-HSA, Cy5, and Cy5-hCG, λ_{max} (exc) = 600 nm, λ_{max} (em) = 660 nm.

Table 1. Fluorescence Decay Times of Sq635-b-butylester (2), Sq635-b-NHS-ester (4), Sq635-b-HSA, Cy5, and Cy5-hCG[a]

sample	decay time (ns)	amplitude	fractional intensity	mean lifetime (ns)	χ^2
Sq635-*b*-butylester (2)	0.21	0.752	0.496	0.43	3.7
	0.65	0.248	0.504		
Sq635-*b*-NHS-ester (4)	0.20	0.558	0.286	0.51	3.8
	0.64	0.442	0.714		
Sq635-*b*-HSA	0.18	0.676	0.142		
	0.96	0.089	0.097	2.26	1.7
	2.81	0.235	0.761		
Cy5	1.01	1	1	1.01	2.1
Cy5-hCG	0.16	0.408	0.071	1.33	2.8
	1.41	0.592	0.929		

[a] Experimental conditions: excitation at 600 nm (rhodamine B dye laser), emission observed through interference filter at 660 nm with 10 nm band; 20 °C (1) The quantum yield of Cy5-avidin (D/P = 2.3) was found to be reduce by 55% on addition of biotinylated BSA.

dye. Figure 8 also reveals the distinct nature of the intensity decays of the free and BSA-bound forms for the squaraine dye and Cy5. The intensity decays of the free and covalently bound Cy5 to hCG (human chorionic gonadropin) are shown for comparison.

DISCUSSION

The synthesized squaraine dyes are highly water soluble, and their conjugates are not likely to precipitate even at high dye-to-protein ratios. The reactive derivatives Sq635-b-NHS-ester (**4**) and Sq635-m-NHS-ester (**9**) exhibit useful fluorescence characteristics and are well suited for covalent attachment to amino groups of proteins. In addition, the new squaraine derivatives can be excited with a laser diode at 635 nm. Their conjugates exhibit high quantum yields relative to the quantum yield of the free dye in water (0.15). This is in contrast to Cy5, a commonly used commercially available protein marker, whose quantum yields are sometimes reduced dramatically in the presence of protein.[2] These features make Sq635-b-NHS-ester (**4**) and Sq635-m-NHS-ester (**9**) attractive alternatives as fluorescent labels in biological assays or biophysical studies of proteins. The high extinction coefficient and quantum yield of the squaraine

dye allowed measurements in whole blood with a detection limit twice as low as Cy5.

We also did an analysis of the frequency domain data for the free and the protein-bound dyes (Table 1). After covalent binding to the protein, the mean fluorescence decay time of the squaraine dye (**2**) increases five times from 0.43 to 2.26 ns. The intensity decay of Sq635-b-butylester (**2**) and Sq635-b-NHS-ester (**4**) measured in water can best be described by two-exponential decays, since the three-exponential models only result in moderate improvement in χ^2 (Table 1). The intensity decay of Sq635-b-BSA is well distinct from those of the free dyes (**2** or **4**). The long component of 2.8 ns contributes 76% to the total fluorescence intensity and thus increases the mean lifetime to 2.26 ns for the protein-bound form. The lifetime increase of the squaraine dyes when covalently bound to proteins can be explained by a shielding effect of the protein. In extreme cases, the dye quasi hides in the hydrophobic pockets of the protein and is protected from the water molecules. In contrast, the mean lifetime of Cy5 increased only from 1.01 to 1.33 ns upon covalent binding to protein (Table 1). These two intensity decays are less distinct as observed for the squaraine dyes (Figure 8) and indicate that Cy5 is less sensitive to the environment than the related squaraine dyes.

CONCLUSION

The synthesis of two reactive water-soluble dyes for derivatization of primary amines and their conjugation procedure to proteins is described. The dyes show absorption and emission maxima in the red region of the visible spectra, which allows the use of inexpensive excitation sources such as diode lasers and avalanche photodiodes as detectors. Long-wavelength absorption and emission is preferred because of the reduced autofluorescence from biological samples in the red region. The dyes also exhibit lower quantum yields and shorter lifetimes in water and improved properties when bound to proteins. We are currently exploring the use of these dyes in lifetime and polarization based immunoassays.

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