

# Red Laser-Induced Fluorescence Energy Transfer in an Immunosystem

Bernhard Oswald,\* Frank Lehmann,\*<sup>1</sup> Lydia Simon,† Ewald Terpetschnig,\* and Otto S. Wolfbeis\*

\**Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, 93040 Regensburg, Germany; and*

†*Central Research Biotechnology, Bayer AG, 51368 Leverkusen, Germany*

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**We describe two near-infrared fluorescent squaraine dyes (Sq635 and Sq660), their spectra, their covalent linkage to proteins, and their use as donor and acceptor, respectively, in a fluorescence resonance energy transfer (FRET) immunoassay based on the use of red lasers. The dyes show quantum yields of around 10% in the free form and up to 68% when bound to proteins. If converted into their *N*-hydroxysuccinimide esters, they can be linked to free amino groups of proteins. To improve water solubility, two sulfo groups were introduced. The emission spectrum of Sq635 overlaps the absorption spectrum of Sq660, a fact that makes them a useful pair of dyes for use in FRET immunoassay which is demonstrated for human serum albumin/anti-human serum albumin.** © 2000 Academic Press

The unique features of diode laser-compatible fluorescent dyes supported their use in biomolecular science in recent years (1, 2). The need for new dyes absorbing and emitting in this wavelength range increased because the optical window of blood and biological tissue is in the wavelength region between 630 and 670 nm. Red diode lasers are inexpensive, cause low autofluorescence of biological material, and reduce damage of the biological samples compared to lasers of shorter wavelengths (3–5).

Fluorescence resonance energy transfer (FRET)<sup>2</sup> is a spectroscopic tool widely used in structural biology,

analytical biochemistry, and polymer science to study the interaction or conformational changes of molecules (1, 2, 6). In FRET, a fluorescent donor transfers its electronic energy nonradiatively to an acceptor which can be fluorescent or nonfluorescent. As a result, the fluorescence intensity of the donor decreases, while the fluorescence of the acceptor increases if the acceptor is fluorescent. FRET can be used to measure distances in the order of 10–100 Å in macromolecules such as DNA, RNA, or proteins (7, 8). Donor and acceptor can be organic dyes, proteins, or chelates.

Ullman *et al.* (9) developed what appears to be the first immunoassay based on FRET by labeling an antigen with fluorescein as the donor dye and an antibody with rhodamine as the acceptor dye. Since then, several attempts have been made to improve FRET-based immunoassays, using labels such as phycobilliproteins or europium chelates (1, 8). The latter, while having long decay times and thus being gated, require short-wavelength excitation and display low extinction coefficients. Others are labile, have poor quantum yields, or—like the Cy dyes (10, 11)—may be quenched by proteins.

We have developed two long-wavelength excitable fluorescent dyes which were covalently bound to the proteins HSA and anti-HSA. As a model system for an immunoassay we examined the conjugates Sq635–HSA/Sq660–anti-HSA and Sq635–anti-HSA/Sq660–HSA.

## MATERIALS AND METHODS

### *Chemicals, Proteins, and Buffers*

Sq635 NHS ester was synthesized following a procedure described recently (10). Sq660 acid was obtained

diolate; Sq660 acid, 2,4-bis[1-(5-carboxypentyl)-3,3-dimethyl-5-sulfonato-2-benzindolinylidene]methylene]cyclobutenediylilium-1,3-

<sup>1</sup> To whom correspondence should be addressed. Fax: 49/941/943-4064. E-mail: Frank.Lehmann@chemie.uni-regensburg.de.

<sup>2</sup> Abbreviations used: anti-HSA, polyclonal anti-human serum albumin; BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; D/P, dye-to-protein ratio; FRET, fluorescence resonance energy transfer; HSA, human serum albumin; mp, melting point; MPLC, middle pressure liquid chromatography; NHS, *N*-hydroxysuccinimide; Sq635 acid, 2,4-bis[1-(5-carboxypentyl)-3,3-dimethyl-5-sulfonato-2-indolinylidene]methylene]cyclobutenediylilium-1,3-

according to (12). HSA and anti-HSA were purchased from Sigma. Sephadex G25 and Amberlite IRC-50 were from Serva; RP C18 silica gel and all other chemicals and solvents were from Merck (Darmstadt). All chemicals were used as received. Phosphate buffer of pH 7.2 (0.1 M) was prepared by dissolving 3.17 g of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  and 13.7 g of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  in 1 L of doubly distilled water. Bicarbonate buffer of pH 9.1 (0.05 M) was obtained by dissolving 2.1 g of  $\text{NaHCO}_3$  in 500 mL of doubly distilled water.

#### Purification and Spectroscopic Analysis

The dyes were purified by MPLC using RP C18 silica gel as the stationary phase and methanol as the eluent. The  $\text{K}^+$  ions of the sulfonic acid groups were replaced by  $\text{H}^+$  ions by ion-exchange chromatography using Amberlite IRC-50 as the stationary phase and methanol as the eluent.

Ion-exchange chromatography of dyes was performed using the weakly acidic cation exchanger Amberlite IRC-50. Gel permeation chromatography was carried out using Sephadex G25 as the stationary phase ( $0.5 \times 20$  cm column) and a 22 mM phosphate buffer solution (pH 7.2) as the eluent. Thin-layer chromatography (TLC) was performed with RP C18 reversed-phase plates from Merck. Melting points 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 with a luminescence spectrometer from Aminco (Series 2, from SLM) with a standard 150-W xenon lamp as the excitation light source.

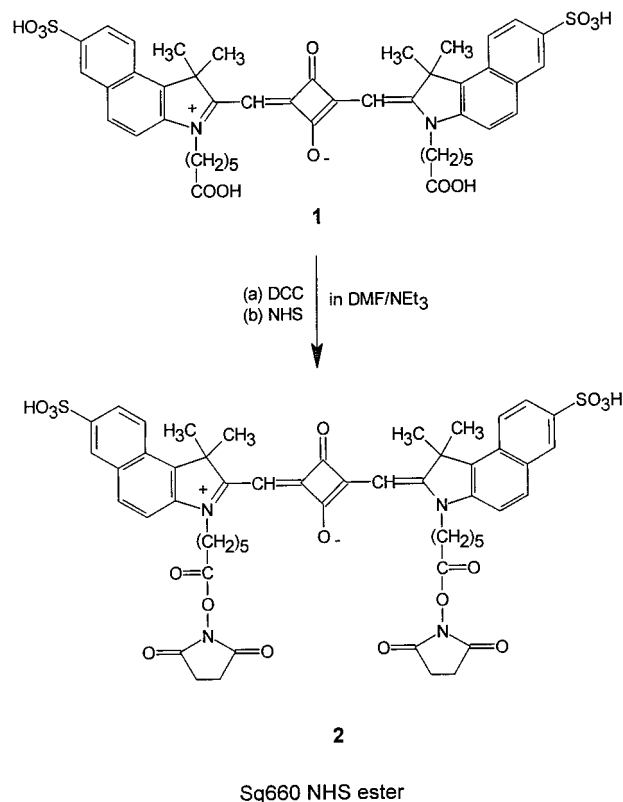
The HSA and anti-HSA concentration was determined using the BCA Protein Assay Reagent Kit from Pierce (Rockford, IL). Quantum yields were determined relative to the dye Cy5 (Amersham, UK) assuming a quantum yield  $\Phi_{\text{Cy5}}$  of 0.28 (13) at  $\lambda_{\text{exc}}$  650 nm for Cy5 using the formula  $\Phi = \Phi_{\text{Cy5}}(F/F_{\text{Cy5}})$ , where  $F$  is  $\int I(\lambda) d\lambda$  (14).

#### Determination of the Extinction Coefficients

The dyes (~0.45 mg) were dissolved in 100 mL of doubly distilled water. Three dilutions (1:25, 1:50, 1:100) were made from this stock solution, and the absorbances were measured. The extinction coefficients were calculated according to Lambert-Beer's law and can be found in Table 1.

#### General Protein-Labeling Procedures and Determination of Dye-to-Protein Ratios

Protein labeling was carried out in a 50 mM bicarbonate buffer of pH 9.1. A stock solution of 1 mg of the NHS ester in 100  $\mu\text{L}$  of anhydrous DMF was prepared



**SCHEME 1.** Activation of Sq660 acid to give Sq660 NHS ester.

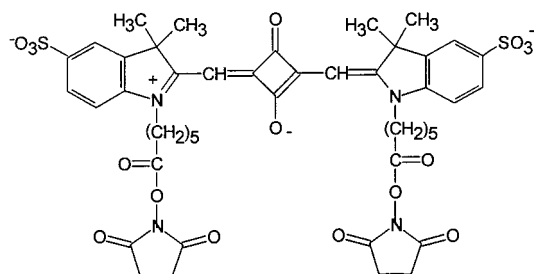
and 5 mg of protein was dissolved in 1 mL of a 50 mM bicarbonate buffer. Various amounts of label from the stock solution were added to the protein solution and the mixture was gently stirred for 5 h (HSA) or for 2 h (anti-HSA) at room temperature in the dark.

Unconjugated label was separated from the labeled proteins using gel permeation chromatography with Sephadex G25 and a 22 mM phosphate buffer of pH 7.2 as the eluent. The fast running fraction (blue in case of Sq635, blue-green in case of Sq660) contained the dye-protein conjugate, while the fraction with a much longer retention time contained the separated free dye.

The degree of labeling (dye-to-protein ratio, D/P) 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 assuming that the  $\epsilon$  of the bound dye is equal to the extinction coefficient of the free dye. The dye-to-protein ratio (D/P) statistically gives the number of labels covalently bound to a single protein.

#### Procedure for Studying Energy Transfer Efficiency

One hundred microliters of the donor solution (Sq635-HSA or Sq635-anti-HSA) in buffer was mixed



**SCHEME 2.** Chemical structure of the Sq635 NHS ester.

with varying quantities of the acceptor solution (Sq660-anti-HSA or Sq660-HSA), typically 5 to 400  $\mu\text{L}$ . The mixtures were diluted with PBS (22 mM, pH 7.2) to a constant end volume of 900  $\mu\text{L}$ . After incubation for 15 min at room temperature, the fluorescence intensity was measured using an excitation wavelength of 600 nm. The slits for both the excitation and emission beam were set to 4 nm.

#### Competitive Immunoassay

The immunoassay experiment was carried out as follows: Constant quantities (100  $\mu\text{L}$ ) of the donor labeled HSA ( $[\text{HSA}] = 2.20 \times 10^{-5}$  M, D/P = 1.0) and various amounts of nonlabeled HSA were mixed at ratios of 1:4, 1:2, 1:1, 1:0.5, 1:0.125, and 1:0 of [Sq635-HSA]:[HSA]. Then, the acceptor labeled anti-HSA ( $[\text{anti-HSA}] = 1.0 \times 10^{-6}$  M, D/P = 1.9) was added in equal amounts (100  $\mu\text{L}$ ). The mixtures were diluted to a constant end volume with PBS (pH 7.2, 22 mM). After an incubation time of 10 min at room temperature, fluorescence was measured at an excitation wavelength of 635 nm using a standard diode laser. The slit of the emission beam was set to 4 nm.

#### Syntheses

*Activation of Sq660 acid to give Sq660 NHS ester.* Twenty-one milligrams (24  $\mu\text{mol}$ ) of Sq660 acid, 12 mg (60  $\mu\text{mol}$ ) of DCC, and 7 mg (60  $\mu\text{mol}$ ) of NHS were

dissolved in 1 mL of anhydrous DMF, and 10  $\mu\text{L}$  of triethylamine was added. The reaction mixture was stirred at room temperature for 20 h and filtered, and the solvent was removed on a rotary evaporator. The blue-green amorphous residue was washed with 5 mL of anhydrous chloroform and 10 mL of anhydrous diethyl ether whereupon the amorphous material crystallizes. The crystals were dried in a desiccator over  $\text{P}_4\text{O}_{10}$ . Yield: 23 mg (88%) of a blue-green crystalline powder. TLC:  $R_f = 0.50$  (on RP-C18, methanol/water = 2/1 (v/v)), mp  $>300^\circ\text{C}$ , FAB-MS,  $m/e$  ( $\text{M}^+$ , dianion) for  $\text{C}_{54}\text{H}_{54}\text{N}_4\text{O}_{16}\text{S}_2$ . Calcd: 1077.2; found: 1077.1.

## RESULTS

### The Squaraine Donor-Acceptor Pair

We have chosen the squaraines as a group of long-wave-absorbing dyes because of their high extinction coefficients ( $>100,000$  L/(mol  $\cdot$  cm)) and their high quantum yields (up to 68%) when covalently linked to proteins which results in a low detection limit of the dye conjugates. Schemes 1 and 2 show the chemical structures of the reactive NHS esters of the donor (Sq635) and acceptor (Sq660). The absorption and emission maxima, molar absorbances, and quantum yields of the dye-protein conjugates are summarized in Table 1. Scheme 1 shows the synthetic pathway for the activation of Sq660 acid to Sq660 NHS ester.

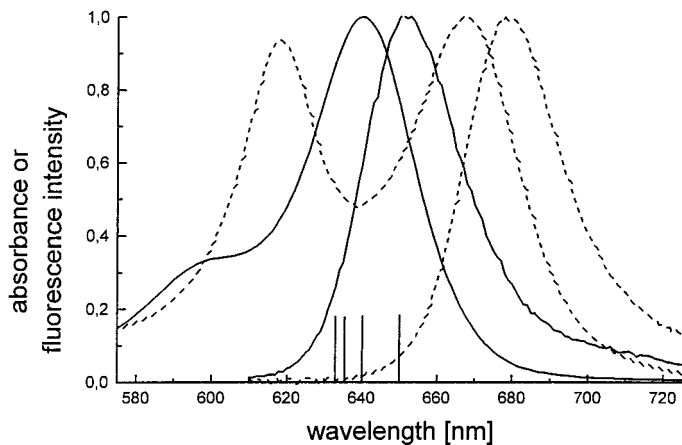
### Spectral Characterization of the Free Donor-Acceptor Dyes

Sq635 NHS ester dissolved in PBS (pH 7.2, 22 mM) has an absorbance maximum at 635 nm and an emission maximum at 642 nm. The excitation wavelength for all emission spectra was 600 nm. For Sq660 NHS ester an absorbance maximum of 660 nm and an emission maximum of 680 nm were found. Details are given in Table 1.

**TABLE 1**  
Spectral Properties and Quantum Yields of Dyes and Dye-Protein Conjugates in PBS of pH 7.2

Dye	Abs. max. [nm]	Em. max. [nm]	$\epsilon$ [L/(mol $\cdot$ cm)]	Q.Y.	D/P [mol/mol]
Sq635	635	642	180,000	0.10	—
Sq635-HSA	642	653	<sup>a</sup>	0.68	0.8
Sq635-anti-HSA	637	648	<sup>a</sup>	0.33	0.9
Sq660	660	680	160,000	0.05	—
Sq660-HSA	672	685	<sup>a</sup>	0.28	4.0
Sq660-anti-HSA	618/668	680	<sup>a</sup>	0.25	4.7
Cy5	647	664	250,000	0.25	—

<sup>a</sup> Not determined.



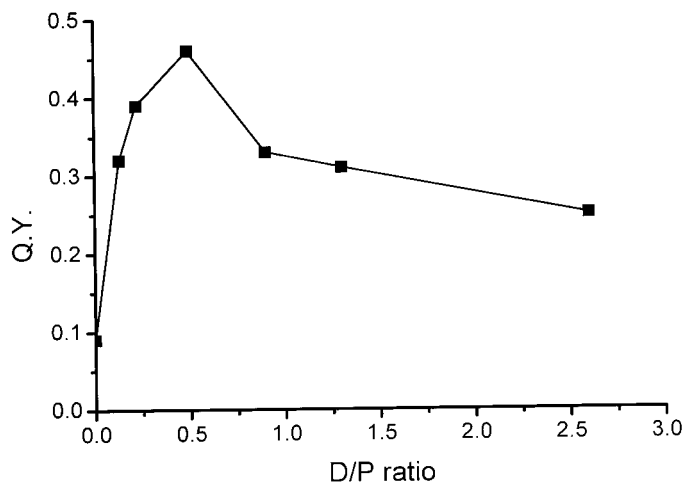
**FIG. 1.** Absorption spectra and emission spectra of Sq635-HSA (—) and of Sq660-anti-HSA (---) ( $\lambda_{\text{exc}} = 600$  nm, 150-W xenon lamp). The vertical lines represent the lines of the 633-nm He/Ne laser and the 635-, 640-, and 650-nm diode lasers, respectively.

### Spectral Characterization of the Protein Conjugates

Figure 1 shows the absorption and emission spectra of Sq635-HSA and Sq660-anti-HSA. The maximum of Sq635 at 642 nm and its emission maximum at 653 nm are slightly shifted toward longer wavelengths when the dye is covalently bound to HSA. Similar spectral shifts are observed for the anti-HSA conjugate (Table 1).

The absorption and emission maxima of Sq660-HSA can be found at 672 and at 685 nm, respectively. Like Sq635, Sq660 shows a bathochromic shift of its absorption and emission maxima in the protein-bound form. Unlike Sq635-HSA, Sq660-HSA shows an additional peak at 621 nm which can be explained by the formation of aggregates (15). The intensity of the dimer absorption peak is increased in the anti-HSA conjugate and is nearly as intense as the maximum at 668 nm.

The intensity of the aggregation peak of Sq660-anti-HSA was found to be independent of factors like ionic strength, temperature, pH, or the dye-to-protein ratio of Sq660-anti-HSA. When the spectrum is taken in a 1/1 (v/v) solution of PBS/ethanol, the dimer intensity at 618 nm is reduced by 45%, while the intensity of the monomer absorption band of 668 nm increases by 50%. Obviously, the fraction of dye aggregates is reduced on addition of ethanol. The same result is obtained on addition of pyridine (16). The isosbestic wavelength is at 655 nm. No change in the absorption spectrum was observed in a PBS solution containing 1% of SDS. Such an aggregation of the dye-protein conjugate can be lowered by increasing the hydrophilicity, e.g., by introducing more than two sulfonic acid groups into the molecule (15).



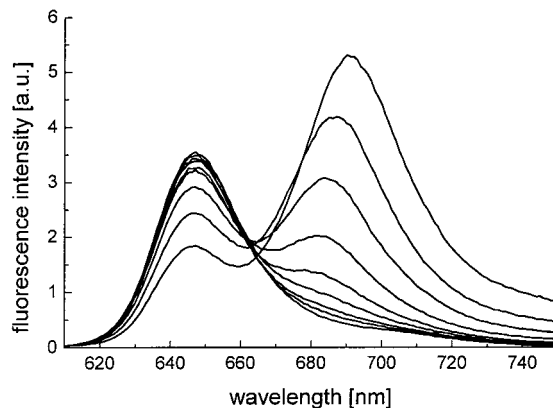
**FIG. 2.** Effect of the dye-to-protein ratio (D/P) on the quantum yield of Sq635-anti-HSA.

### Quantum Yields

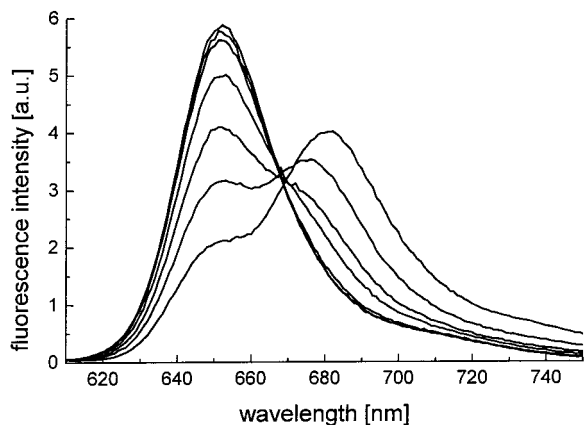
The dependence of the quantum yield on the D/P ratio of Sq635-anti-HSA is shown in Fig. 2. The quantum yield of the free dye (0.09) increases on labeling the protein and reaches a maximum of 0.46 for a D/P ratio of 0.5. Even higher quantum yields can be obtained for the HSA conjugates: the maximum quantum yield determined for the Sq635-HSA conjugates is 0.68 (D/P 0.9).

### Energy Transfer Studies and Immunoassay

A representative energy transfer measurement is shown in Fig. 3: Sq635-anti-HSA (D/P = 3.2, [anti-HSA] =  $1.0 \times 10^{-6}$  M) was mixed with various amounts of Sq660-HSA (D/P = 4.0) in the molar ratios of 1:8, 1:4, 1:2, 1:1, 1:0.5, 1:0.25, 1:0.125, and 1:0. The fluores-



**FIG. 3.** Energy transfer study in which Sq635-anti-HSA (D/P = 3.2) of constant concentration was titrated with Sq660-HSA (D/P = 4.0) ( $\lambda_{\text{exc}} = 600$  nm). [anti-HSA] =  $1.0 \times 10^{-6}$  M, molar ratios are 1:8, 1:4, 1:2, 1:1, 1:0.5, 1:0.25, 1:0.125, 1:0.

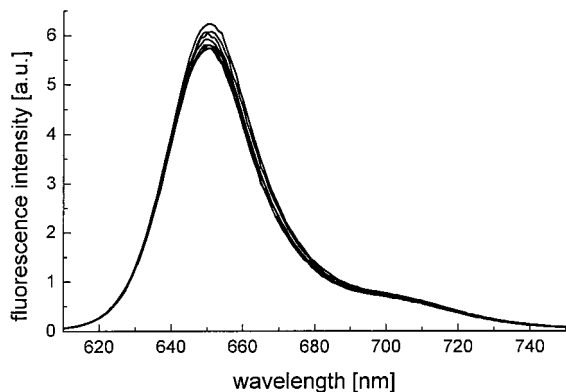


**FIG. 4.** Energy transfer study where Sq635-HSA (D/P = 0.8) of constant concentration was titrated with Sq660-anti-HSA (D/P = 4.7) ( $\lambda_{\text{exc}} = 600$  nm). [HSA] =  $2.2 \times 10^{-6}$  M, molar ratios are 1:4, 1:2, 1:1, 1:0.5, 1:0.25, 1:0.125, 1:0.

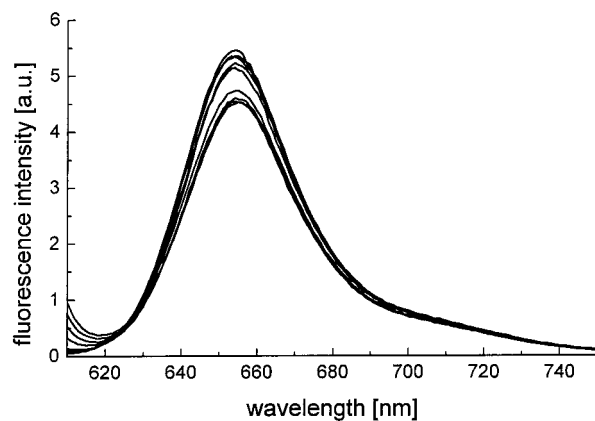
cence intensity of Sq635-anti-HSA decreases with increasing quantities of Sq660-HSA, while the fluorescence intensity of Sq660-HSA increases due to energy transfer (Fig. 3).

Figure 4 shows the energy transfer for a system with switched donor and acceptor. Sq635-HSA (D/P = 0.8, [HSA] =  $2.2 \times 10^{-6}$  M) was mixed with various amounts of Sq660-anti-HSA (D/P = 4.7) in the molar ratios of 1:4, 1:2, 1:1, 1:0.5, 1:0.25, 1:0.125, and 1:0. The fluorescence intensity of Sq635-HSA decreases with increasing amounts of Sq660-anti-HSA, while the fluorescence intensity of Sq660-anti-HSA increases due to energy transfer. The excitation wavelength was also 600 nm. The Förster distance  $R_0$  was determined to be 70 Å in both cases. At  $R_0$ , one half of the donor decays by energy transfer and the other decays by the usual radiative and nonradiative rates.

In Figs. 5 and 6 the donor was titrated with nonlabeled HSA or anti-HSA, respectively, to show that the



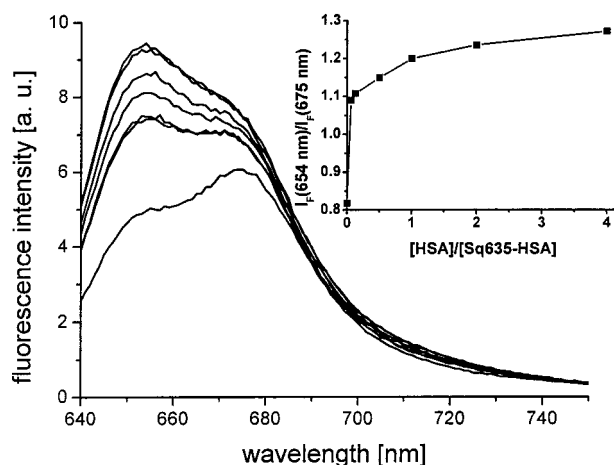
**FIG. 5.** Titration of Sq635-anti-HSA with nonlabeled HSA ( $\lambda_{\text{exc}} = 600$  nm).



**FIG. 6.** Titration of Sq635-HSA with nonlabeled anti-HSA ( $\lambda_{\text{exc}} = 600$  nm).

decrease of the donor fluorescence is not due to quenching of the proteins but due to energy transfer. The energy transfer was found to be independent of pH in the range of 6–8; further additional BSA did not influence the energy transfer either. It was found that the optimal conditions for the energy transfer are the following: D/P (donor)  $\sim 1.1$ –3.0, D/P (acceptor)  $\sim 1.6$ –4.2, protein concentration  $\sim 1 \times 10^{-6}$  to  $7 \times 10^{-6}$  M. The detection limits of the proteins are at about  $1 \times 10^{-7}$  M which corresponds to about 7  $\mu\text{g/mL}$  for HSA and 16  $\mu\text{g/mL}$  for anti-HSA.

Figure 7 demonstrates two important features: First, the red laser compatibility with a standard 635 nm diode laser is shown. Second, a competitive immunoassay is presented, where the concentration of free HSA can be determined from the ratio of the donor fluorescence intensity and the acceptor fluorescence intensity. The donor is Sq635-HSA and the acceptor is Sq660-



**FIG. 7.** Competitive immunoassay with Sq635-HSA/Sq660-anti-HSA and free HSA as the antigen to determine. Excitation wavelength is 635 nm using a standard diode laser.

anti-HSA. The fluorescence intensity was measured and the ratios of the two emission maxima at 654 nm (donor) and at 675 nm (acceptor) were determined. In the inset of Fig. 7 the ratio of the intensities is plotted against the ratio of nonlabeled and labeled HSA and shows a saturation curve. The saturation is reached at a ratio of 4:1.

## DISCUSSION

Labels Sq635 and Sq660 represent two water-soluble and reactive fluorophores whose quantum yields increase substantially when covalently bound to proteins. Due to their strong absorptions between 600 and 700 nm, they are excitable by red diode lasers. The high quantum yields and extinction coefficients along with their availability as reactive NHS esters make them viable labels for proteins, particularly for use in fluorescence energy transfer immunoassay. Unlike Cy dyes, the squaraine dyes are not quenched by proteins. In contrast, their fluorescence increases in presence of proteins. In contrast to allophycocyanins (another class of red laser excitable fluorophores), the squaraine labels have the advantage of rather low molecular weight.

The detection limits of free and anti-HSA-labeled Sq635 NHS ester were compared to those of free and HSA-labeled Cy5 NHS ester at concentrations of 50 nM in human blood diluted 1:20 with PBS. The Sq dye could be detected in concentration as low as 10 nM which is lower by a factor of 2 compared to Cy5, which is mostly due to its higher quantum yield (10).

The energy transfer studies using two squaraine dyes demonstrate that the dyes show suitable features such as good spectral overlap and high quantum yields (donor) as well as high extinction coefficients (acceptor) which results in a high  $R_0$  of 70 Å. Both combinations of conjugates, Sq635-HSA/Sq660-anti-HSA and vice versa were used in the energy transfer studies. The detection limits of the proteins in the energy transfer studies were determined to be at  $1 \times 10^{-7}$  M using a 150-W standard xenon lamp. The titration sequence plays an important role in our energy transfer experiments. Positive results were only obtained if the donor-labeled protein was titrated at fixed concentrations with varying quantities of acceptor-labeled conjugates. If this sequence is switched, i.e., the acceptor is titrated at a constant concentration with various amounts of donor, no energy transfer could be observed. This phenomenon cannot be explained yet and will be further investigated. Notwithstanding, the titration of donor with acceptor leads to highly reproducible results.

The immunoassay study shows that the dyes are suited for homogenous competitive immunoassays in which an antigen concentration can be determined by ratioing the intensity at two wavelengths.

## ACKNOWLEDGMENTS

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