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An Investigation of Squaraines as a New Class of Fluorophores with Long-Wavelength Excitation and Emission

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We present an overview of our investigation of the spectral properties and photostabilities of squaraine derivatives. The objective was to identify long-wavelength probes with reasonable quantum yields, reasonably long lifetimes, and good photostabilities for use in fluorescence-based assays and/or imaging. Both symmetrical and unsymmetrical squaraines were studied. Based on this investigation the most suitable probes for use in a biological application were found to be the symmetrical indolenine derivatives of the squaraines, which display the highest photostability. Importantly, their quantum yields and lifetimes increase significantly upon covalent and noncovalent binding to proteins (bovine serum albumin, antibodies), demonstrating the usefulness of the squaraines as long wavelength probes. The squaraine absorbance maxima between 630 and 650 nm allows the use of the new commercially available 635- and 650-nm diode lasers.

KEY WORDS: Fluorescence sensing; squaraines; phase-modulation fluorometry; red fluorescence; near-infrared fluorescence.

INTRODUCTION

Fluorescence detection is widely used in immunoassays and fluorescence microscopy, and there is an increasing use of fluorescence in analytical and clinical chemistry [1–3]. In such measurements it is advantageous to use long-wavelength excitation and emission, which results in decreased autofluorescence from cells and tissues and allows the use of simple laser light sources. However, the use of simple lasers, such as the 635- to 670-nm laser diodes, has been hindered by the lack of suitable fluorescent probes.

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RESULTS AND DISCUSSION

We reported previously the chemical synthesis and spectral properties of squaraines for use as long wavelength-emitting probes in fluorescence-based assays [4,5]. For this purpose 10 squaraines were synthesized with different combinations of their heterocyclic nuclei. The chemical structures of the synthesized squaraines, as well as the structure of the commercially available CY-5.18-NHS, are shown in Fig. 1. These dyes have structures that are similar to those of cyanine dyes but also contain a central squarate bridge. The squarate residue shifts the absorption and emission maxima to longer wavelengths, relative to the comparable cyanine dye, and is expected to increase the photostability of the dyes [6].

Based on this investigation [4] the most photostable squaraines were found to be the indolenine dyes. Importantly, these squaraines also display the largest in-

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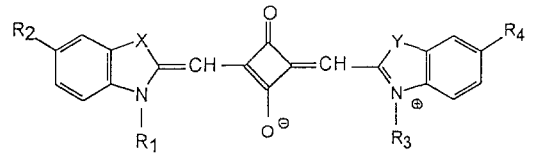
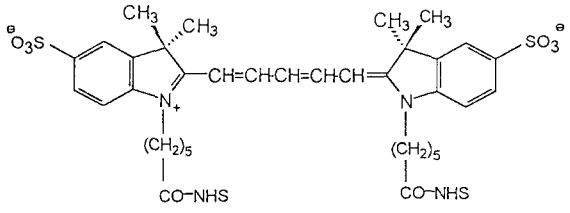


Compound	X	Y	R_1	R_2	R_3	R_4
1	$C(Me)_2$	$C(Me)_2$	Et	H	Et	H
2	S	S	Et	H	Et	H
3	Se	Se	Et	H	Et	H
4	$C(Me)_2$	$C(Me)_2$	Me	Cl	Me	Cl
5	$C(Me)_2$	S	Me	H	Et	H
6	$C(Me)_2$	Se	Me	H	Et	H
7	$C(Me)_2$	S	Me	Cl	Et	H
8	$C(Me)_2$	$C(Me)_2$	Me	Cl	Me	H
9	$C(Me)_2$	Se	Me	Cl	Et	H
10	S	Se	Et	H	Et	H
Sq-NHS	$C(Me)_2$	$C(Me)_2$	Et	CO-NHS	Me	Cl
SbSq-NHS	$C(Me)_2$	$C(Me)_2$	$(CH_2)_4SO_3^-$	CO-NHS	Me	Cl



CY-5.18-succinimidylester

Fig. 1. Chemical structures of the synthesized squaraines (top) and a comparable CY5.18-NHS ester (bottom).

crease in lifetime and quantum yield upon binding to bovine serum albumin (BSA) (about 15-fold) compared to MeOH (Fig. 2). The nanosecond lifetimes displayed by the indolenine derivatives when bound to BSA are adequately long to allow the use of simple phase-modulation instrumentation, which can be practical in a clinical environment [7]. We concluded that the absorption and emission spectral properties, and the lifetimes, of the indolenine derivatives of squaraines were suitable for their use as labels in immunoassays and other clinical applications.

To couple covalently the squaraine fluorophore to antibodies, we synthesized two *N*-hydroxysuccinimide ester (NHS) derivatives of the squaraines, which are reactive with the amino functions of such biomolecules [8]. First, a water-insoluble form, which lacks the sulfobutyl group, was synthesized. This water-insoluble Sq-NHS was recated with the amino function of taurine (2-aminoethanesulfonic acid) to achieve water solubility for the purpose of testing its spectral properties in aqueous media. The Sq-taurine derivative appeared to have a very high affinity for BSA. The fluorescence lifetimes and relative quantum yields of the Sq-taurine derivative in water increased dramatically in the presence of BSA. In this regard the squaraines are comparable to the classical fluorophores ANS and TNS, which display minimal fluorescence in water but are highly fluorescent when bound to BSA and other proteins [9,10].

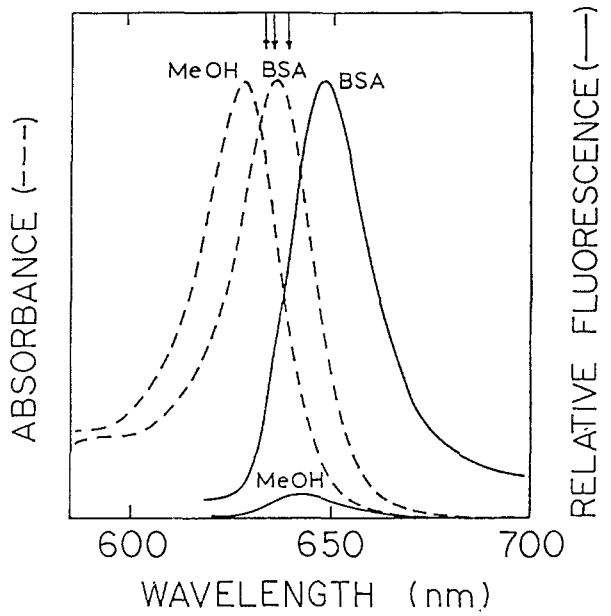


Fig. 2. Absorption (- - -) and relative emission spectra (——) of indolenine–squaraine 1 in MeOH and in the presence of 5 mg/ml BSA. Arrows indicate lines of available laser sources for excitation (red HeNe, 633 nm; diode lasers, 635 and 640 nm).

Due to its water insolubility the conjugation of the Sq-NHS to an antibody caused the antibody conjugate to precipitate, in particular at higher dye-to-protein (D/P) incorporation ratios. This forced us to synthesize a water-soluble (sulfobutyl group-containing) form of the NHS ester (SbSq-NHS), which would display the desirable spectral properties without the disadvantage of reducing the water solubility of the conjugates. Figure 3 and 4 illustrate the properties of the squaraine probe and its antibody conjugates in comparison to those of the commercially available CY5 reactive dye. The quantum yield of the free squaraine fluorophore in water is very low (ϕ = 0.025) but increases six times (ϕ = 0.16) upon conjugation to the antibody, thus reducing the contribution of free dye to the fluorescence signal (Fig. 3).

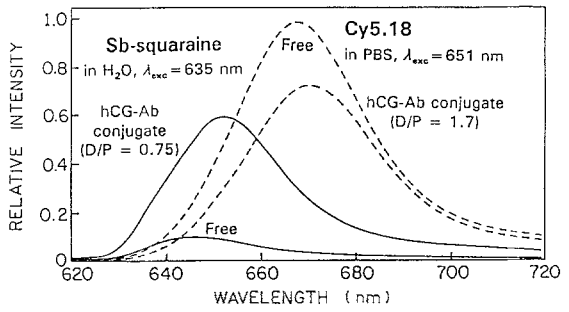


Fig. 3. Relative emission spectra of sulfobutyl-squaraine (SbSq-OH) in water (free) and conjugated to hCG-antibody. Dashed lines indicate relative emission spectra of CY5.18-OH in PBS (free) and conjugated to hCG antibody.

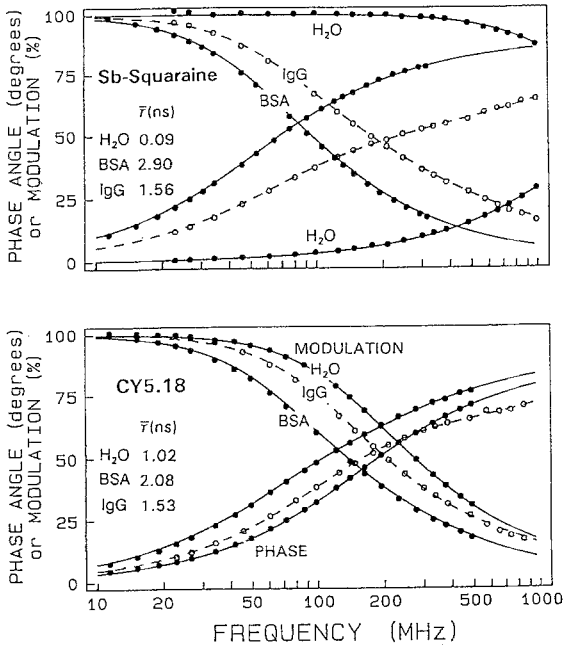


Fig. 4. Frequency responses of intensity decays and mean lifetimes of Sb-squaraine (top) and CY5.18 (bottom) in water (H_2O, in 5 mg/ml BSA (BSA), and conjugated to hCG-antibody (IgG).

The increase in lifetime and shift in frequency response upon binding to proteins introduce a much smaller contribution of the free dye to intensity on lifetime measurements of the squaraine probe compared to CY5 (Fig. 4).

We compared the photostability of SbSq-OH with that of CY5.18-OH, the precursor of the commercially available NHS ester. The photostabilities of the SbSq-OH and CY5.18-OH are comparable. Photobleaching for the SbSq-OH is about 20% less in BSA solution than in water (Fig. 5). Increased photostability in the presence

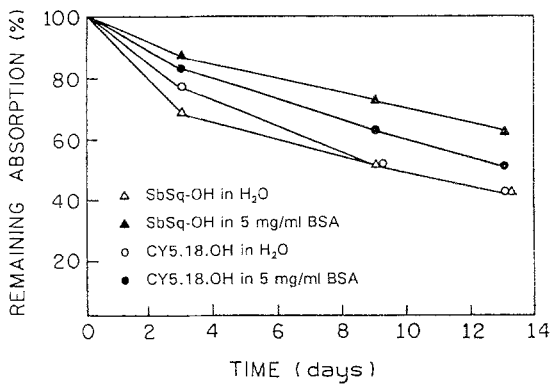


Fig. 5. Remaining absorption (%) vs time (days) of exposure to light of SbSq-OH in water and 5 mg/ml BSA, compared to CY5.18-OH.

of protein is desirable, in particular, for reactive dyes, which are used for covalent attachment to proteins.

CONCLUSION

This investigation reveals the valuable properties of squaraines of increasing the quantum yield and lifetime upon binding to proteins, making them a good choice for a conjugatable fluorescent label in immunochemical assays or biophysical studies of proteins.

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REFERENCES

1. O. S. Wolfbeis (Ed.) (1991) *Fiber-Optic Chemical Sensors and Biosensors, Vols. I and II*, CRC Press, Boca Raton, FL.
2. K. Van Dyke (Ed.) (1990) *Luminescence Immunoassay and Molecular Applications*, CRC Press, Boca Raton, FL.
3. O. S. Wolfbeis (Ed.) (1993) *Fluorescence Spectroscopy—New Methods and Applications*, Springer-Verlag, Berlin.
4. E. Terpetschnig, H. Szmacinski, and J. R. Lakowicz (1993) *Anal. Chim. Acta* **282,** 633–641.
5. E. Terpetschnig and J. R. Lakowicz (1993) *Dyes Pigments* **21,** 227–229.
6. S. Yasui, M. Matsuaka, and T. Kitao (1988) *Dyes Pigments* **10,** 13.
7. J. R. Lakowicz (1993) *Laser Focus World* **28**(5), 60–80.
8. E. Terpetschnig, H. Szmacinski, A. Ozinskas, and J. R. Lakowicz (1993) *Anal. Biochem.* **217,** 197–204.
9. J. Slavik (1982) *Biochim. Biophys. Acta* **694,** 1–25.
10. G. M. Edelman and W. U. McClure (1968) *Acc. Chem. Res.* **1,** 65–70.