Molecular Probes

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A Near-Infrared Squaraine Dye as a Latent Ratiometric Fluorophore for the Detection of Aminothiol Content in Blood Plasma**

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Dedicated to Professor M. V. George on the occasion of his 80th birthday

The design of molecular probes (e.g., chemosensors and chemodosimeters) is a priority research topic because of their wide range of applications in the broad area of chemistry and biology.[1] Among the variety of possible detection methods, the technique of fluorescence spectroscopy is the most sensitive for the detection of a specific analyte. Chemodosimeters can show irreversible fluorescence quenching or color bleaching for one-time detection of an analyte.[2] However, these strategies are not the best methods of detecting the response of an analyte for many reasons. In some cases the fluorescence signal is strongly influenced by other competing analytes or even by minor impurities. Similarly, bleaching of color can also arise from chemical changes induced by impurities rather than the target molecules. These potential limitations pose serious threats to the sensitivity and selectivity of a given probe. Therefore, researchers have focused on molecular probes that generate an intensely absorbing or emitting species upon interaction with an analyte, and that allow ratiometric probing and imaging at wavelengths different from that of the original probe. Moreover, the practical application of a given probe is of prime importance.

Squaraine dyes[3,4] have been extensively investigated as chemosensors[5–7] and chemodosimeters.[8] Recently, Ros-Lis et al. reported the use of a squaraine dye for the detection of thiols, which was based on color bleaching and fluorescence quenching.[8b] However, the absorption and emission spectra of squaraine dyes are strongly influenced by acidic and basic impurities, polarity, and the pH value of the surrounding medium. Therefore, it is desirable to have a probe that is stable and produces a strongly absorbing or emitting species in the visible/NIR (NIR = near-infrared) region upon interaction with a specific analyte, rather than the usually encountered color bleaching or fluorescence quenching mechanisms that allow ratiometric detection. Herein, we report a π-extended squaraine dye that exhibits remarkable absorption and emission changes in the presence of aliphatic thiols and demonstrate its application in the detection of low-molecular-weight amino thiols such as cysteine and homocysteine in human blood plasma (HBP). The present strategy involves the chemical activation of a weakly fluorescent NIR probe through a conjugation break upon nucleophilic attack by a thiol such that a strongly emitting fluorophore is generated.

Cysteine (Cys) and homocysteine (Hcy) are thiol-containing amino acids present in human blood. Epidemiological studies have shown that an excess of Hcy in the blood plasma can lead to a higher risk of coronary heart disease, stroke, and peripheral vascular diseases.[9] Other evidence suggests that Hcy may have an effect on atherosclerosis by damaging the inner lining of arteries and promoting blood clots.[10] One of the factors that influences the level of aminothiols in blood plasma is smoking.[11] The atherogenic property of tobacco smoking is connected with its influence on Hcy and thereby the total aminothiol concentration in HBP.[12] Therefore, detection of thiols in biological samples is important.[13] In this context, the development of new, simple, and efficient methods for the determination of the total aminothiol content in blood plasma is of considerable significance.

The reaction of a strongly fluorescent bispyrrole (λ_{em} = 470 nm in acetonitrile, $\Phi_f = 0.43$, quinine sulfate as standard) with squaric acid (2:1 stoichiometry) using a Dean–Stark apparatus resulted in a weakly fluorescent NIR dye **Sq1**.[14] The dye was purified by column chromatography and characterized by NMR and MS analyses.[14b,d] The UV/Vis spectrum of **Sq1** in acetonitrile/water (1:1) solution shows bands at 750 nm ($\varepsilon = 1.8 \times 10^5$) and 363 nm (Figure 1a). The emission spectrum of **Sq1** exhibits a weak band at 800 nm (λ_{ex} = 730 nm). In analogy with previous reports, we speculated that aliphatic thiols may add on the cyclobutene ring and shift the double bond that connects the cyclobutene ring with the pyrrole moiety in **Sq1**.[8,15] Thus, regeneration of the initial chromophores, which breaks the electronic communication between the two bispyrrole moieties within the probe itself, is possible (Scheme 1). In this way the NIR absorption of the probe is shifted to visible wavelengths and results in strong emission. This was established by the addition of aliphatic thiols or a thiol-containing α-amino acid cysteine (0–4.9×10^{-4} M) to **Sq1** (6×10^{-6} M) in 1:1 acetonitrile/water (2-(cyclo-

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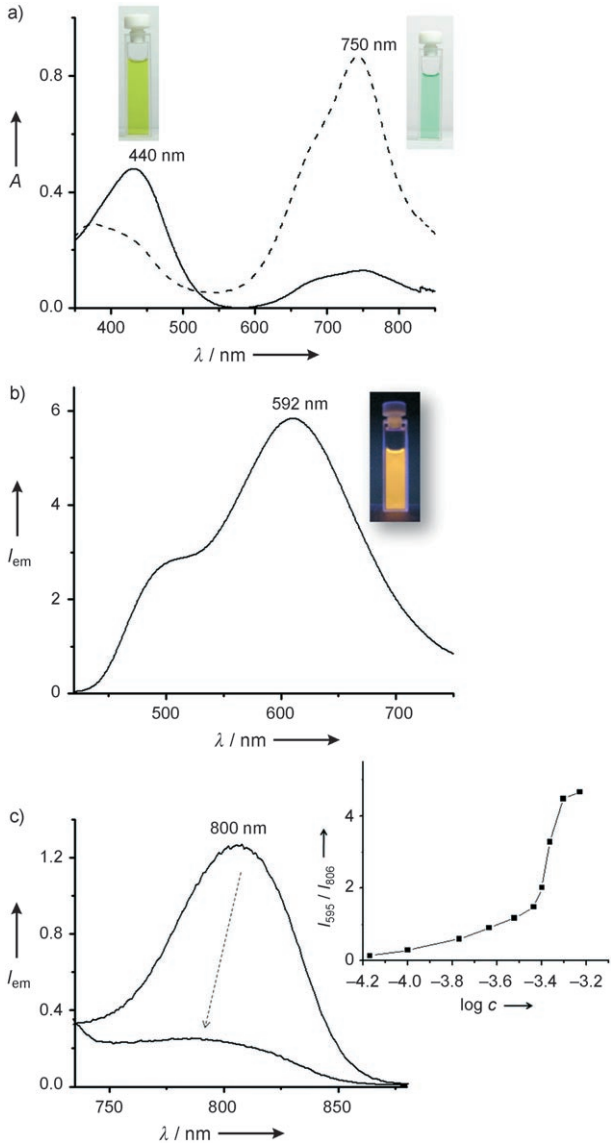
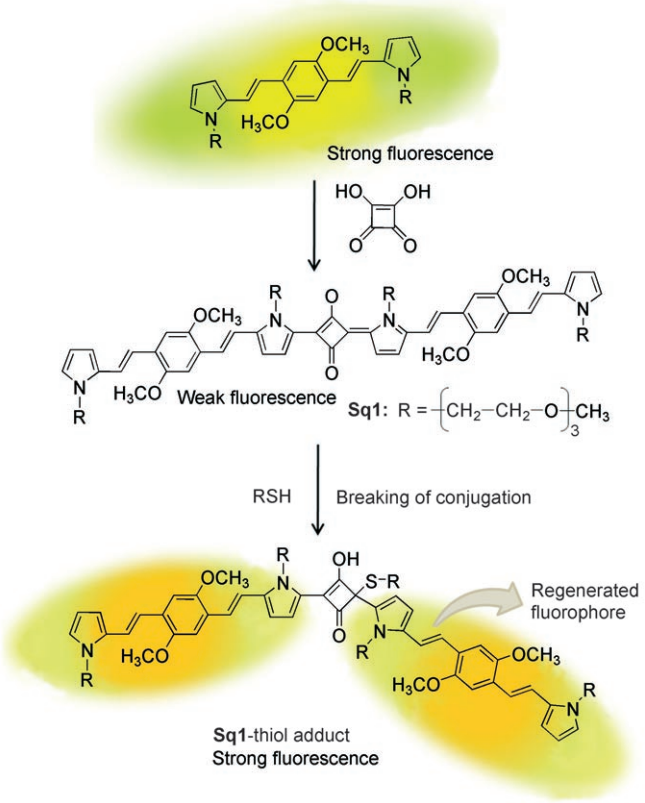


Figure 1. a) UV/Vis spectra of **Sq1** [6×10^{-6} M] before (•••••) and after (——) the addition of one equivalent of cysteine in acetonitrile/water (1:1, CHES buffer (0.01 M), pH 9.6). b) and c) Emission spectra of the **Sq1**–cysteine adduct when excited at 410 nm and 730 nm, respectively. A ratiometric plot of the emission intensity changes at 595 nm and 806 nm against cysteine concentration is shown as an inset.



Scheme 1. Chemical activation of a weak fluorophore **Sq1** to an active fluorophore through thiol group attack. The background color indicates the fluorescence color of the molecules.

hexylamino) ethanesulfonic acid (CHES) buffer, pH 9.6) which resulted in a reduction in the intensity of the absorption band at 750 nm and concomitant formation of a new band at 440 nm. The reaction of **Sq1** with thiol groups can be followed visually by a color change from green to light yellow (Figure 1 a).

The emission properties of the dye **Sq1** showed a dramatic change upon addition of cysteine. When excited at 410 nm, the emission spectrum of the **Sq1**–cysteine adduct exhibited a new band at 592 nm with bright orange fluorescence (Figure 1 b), whereas a decrease in the intensity of the weak NIR emission at 800 nm ($\lambda_{ex}=730$ nm) with increasing concentration of cysteine upon was observed (Figure 1 c). These changes in the absorption and emission spectra are attributed to the addition of the thiol group to the cyclobutene ring, which results in the formation of new strongly fluorescent chromophores. The noninterfering absorption bands with large wavelength shift ($\Delta\lambda=310$ nm) and the possibility to monitor two emission maxima at dual wavelength excitation make the new molecule a unique ratiometric probe (Figure 1 c inset and Figure S5 in the Supporting Information).[16–18]

Addition of different amino acids without thiol groups to **Sq1** did not alter its absorption or emission properties, which indicated the selective interaction of the thiol group in cysteine. This is clear from Figure 2, which shows plots of the intensity of the emission at 592 nm against the corresponding amino acids. Thus, the two non-interfering absorption bands and the possibility of monitoring the emission at two different excitation wavelengths extend the scope of **Sq1** as a suitable ratiometric molecular probe for detection of thiol-containing amino acids in biological samples, especially in human blood plasma (HBP).

Most of the thiol-containing amino acids in HBP such as cysteine and homocysteine are in the disulfide form, bound either to protein or to low-molecular-mass thiols. Estimation of the aminothiol level in blood plasma is essential for understanding the role of these groups in the pathogenesis of vascular diseases. In this context, we investigated the use of **Sq1** in the evaluation of the total content of aminothiols in HBP under different conditions. Addition of aliquots (10, 20, 30, and 40 μL) of HBP after reduction (see Experimental

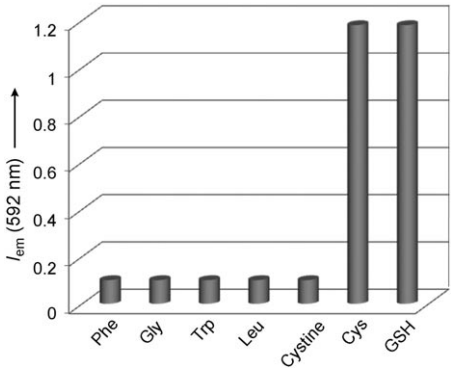


Figure 2. Plot of the response of **Sq1** (6×10^{-6} M) to different amino acids and to a tripeptide glutathione (GSH). The emission intensity is monitored at 592 nm in acetonitrile/water (1:1, CHES buffer (0.01 M), pH 9.6). Amino acids without a thiol moiety as well as cystine (two cysteine molecules joined by a disulfide bond) did not show any response.

Section) to **Sq1** (in water/acetonitrile 1:1, pH 9) resulted in a decrease of the absorption band at 750 nm with the formation of a new band at 440 nm. Consequently, the intensity of the emission band of **Sq1** at 800 nm decreased, with the formation of a new emission band at 600 nm (Figure 3), which indicates the presence of free aminothiols that form fluorescent adducts with the probe.

Microtest assay experiments for the detection of thiol-containing amino acids and low-molecular-weight aminothiols in HBP were conducted in microwell containers, each filled with 200 μL of a solution of **Sq1** in water/acetonitrile (1:1) at pH 9.6 (CHES buffer (0.01M), Figure 4). Equal volumes of different amino acids were separately added to the wells (A–C). The samples in the microwells were illuminated from underneath by 365 nm UV light in a gel documentation apparatus. Interestingly, only L-cysteine (C1) and reduced glutathione (C3) exhibited intense emission (orange) at 600 nm. The fourth row (D) shows the response of **Sq1** to aliquots of HBP under different conditions. The bright

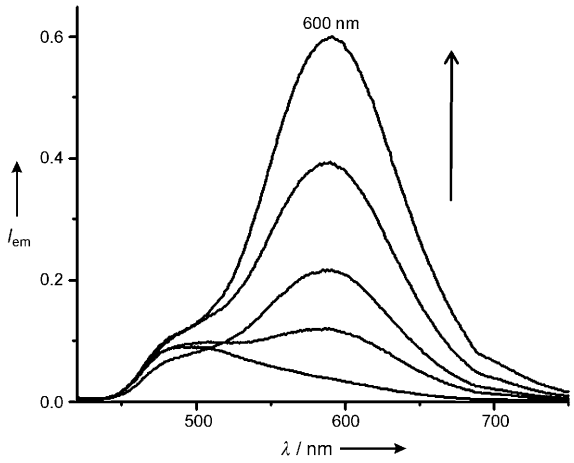


Figure 3. a) Increase in the emission intensity of **Sq1** (6×10^{-6} M) at 600 nm ($\lambda_{ex}=410$ nm) upon addition of aliquots (0, 20, 30, 40 μL) of human blood plasma after reduction.

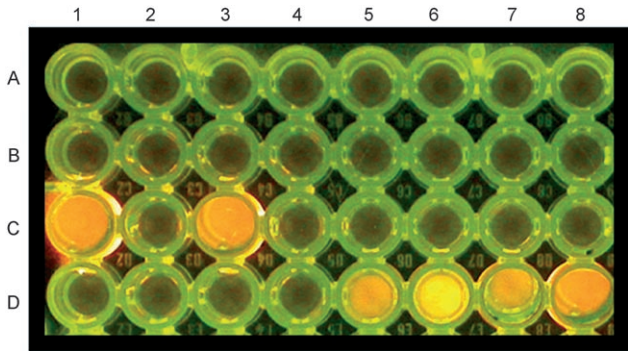


Figure 4. Detection of aminothiols in human blood plasma using a microwell test assay. Contents of wells (each contain **Sq1**): Well A1 (blank, **Sq1** alone), A2 (phenylalanine), A3 (threonine), A4 (arginine), A5 (histidine), A6 (asparagine), A7 (leucine), A8 (alanine), B1 (proline), B2 (valine), B3 (glycine), B4(lysine), B5 (serine), B6 (methionine), B7 (isoleucine), B8 (glutamine), C1 (L-cysteine), C2 (cystine), C3 (glutathione), C4 (tryptophan), C5 (glutamic acid), C6 (aspartic acid), C7 (aqueous buffer at pH 6), C8 (aqueous buffer at pH above 10), D1 (**Sq1** alone), D2 (20 μL solution from blank experiment conducted without HBP), D3 (10 μL HBP before reduction), D4 (20 μL HBP before reduction), D5–D8 (different HBP samples after reduction).

orange emission indicating the presence of aminothiols was obtained only in microwells D5–D8, which contained different HBP samples after reduction.

The biological application of **Sq1** is demonstrated by the quantitative estimation of aminothiols in HBP. It is known that the total aminothiol content in plasma varies for many reasons.[9–11] For example, aminothiol concentration in blood before and after breakfast, as well as after smoking, is known to vary significantly. Therefore, quantitative determination of free aminothiols is of great importance. Analysis of HBP samples collected under three different physiological conditions by using **Sq1** revealed significant variation in the aminothiol content. The unknown concentrations of aminothiols were determined by the standard addition method using cysteine as the standard.[19] For comparison of the total aminothiol concentration with dietary intake, blood samples were analyzed before breakfast (BBF) and after breakfast (ABF). To study the effect of tobacco smoking on aminothiol production, a third sample was taken from a person who smokes between eight and ten cigarettes per day (see Experimental Section and the Supporting Information for details). HBP before breakfast was taken as the standard and compared with the other samples after reduction of the protein-bound aminothiols. Analysis of the reduced HBP using **Sq1** revealed that the amount of total aminothiol concentration is high in the HBP after breakfast, which arises from the physiological processes associated with dietary intake (Figure 5).[12a,20] However, the plasma sample (300 μL) obtained after smoking showed very high aminothiol content of 545 $\mu g\,L^{-1}$. Thus, **Sq1** is a suitable molecular probe for the detection and estimation of the total aminothiol level in HBP, and thus has significant biological and clinical relevance.

In conclusion, we have demonstrated a novel strategy for the detection of low-molecular-weight amino thiols by using a π-extended squaraine dye that exhibits a NIR/Vis electronic

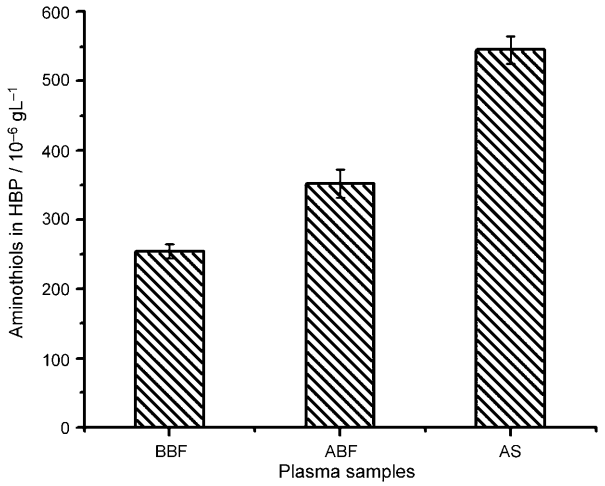


Figure 5. Plots showing the variation in the amount of total aminothiol concentrations in 300 μL HBP obtained at three different conditions. BBF (HBP from a person before breakfast), ABF (HBP from a person after breakfast), and AS (HBP from a person after smoking).

transition. **Sq1** is a novel ratiometric probe which is different from other known probes as detection is based on the generation of a new fluorophore that emits at a different wavelength through an analyte-induced breaking of π-conjugation. This is particularly important in biolabeling and imaging applications since the presence of any unchanged probe does not interfere with the measurement as the native dye is nearly nonfluorescent. The present system is also reliable as a chromogenic probe because the analyte interaction leads to a color formation rather than a color bleaching. The probe was successfully used for the detection and estimation of aminothiols in human blood plasma and confirmed the effect of smoking on the increased level of aminothiols in blood. The present probe may have further application in the fluorescent labeling and imaging of thiol-containing proteins in biological fluids.

Experimental Section

Fresh human blood samples (5 mL) with added ethylenediaaminetetraacetic acid (EDTA) were centrifuged in a vacutainer tube at 3000 rpm for 15 min. The supernatant solution (plasma), which contains proteins and amino acids, was collected and used as such for further studies. For a healthy adult, blood is composed of about 43–56% blood plasma. 500 μL of collected plasma was reduced using dilute HCl in the presence of triphenylphosphine as catalyst. Proteins present in the sample after reduction were precipitated by the addition of acetonitrile, followed by centrifugation (6000 rpm) of the sample for 20 min. The supernatant liquid, which contained aminothiols in blood plasma, was filtered through a nylon filter and diluted to 2 mL with water/acetonitrile (1:1) and used as such for analysis. Aliquots of the plasma sample were added to solutions of **Sq1** (6×10^{-6} M) in acetonitrile/water 1:1 (CHES buffer 0.01M, pH 9.6) and the emission at 600 nm was determined. The unknown amount of aminothiols was estimated using the standard addition method.

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