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Chloride-Sensitive Fluorescent Indicators

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Three fluorescent halide-sensitive quinolinium dyes have been produced by the reaction of the 6-methylquinoline heterocyclic nitrogen base with methyl bromide, methyl iodide, and 3-bromo-1-propanol. The quaternary salts, unlike the precursor molecule, are readily water soluble and the fluorescence intensity of these salts is reduced in the presence of aqueous chloride, bromide, and iodide ions, allowing halide solution concentrations to be determined using well-known Stern–Volmer kinetics. One of the dyes, dye 1, has a chloride Stern–Volmer constant of 255 mol^{-1} dm^3 which is more than twice that of SPQ [6-methoxy-*N*-(3-sulfopropyl)quinolinium] used in recent physiological measurements to measure intracellular chloride levels. The dyes have been characterized using steady-state fluorescence spectroscopy and are compared to three similar dyes based on the 6-methoxyquinoline nucleus, reported earlier by the authors, and also to dyes reported by Krapf *et al.* (*Anal. Biochem.* 169, 142–150, 1988). The interference of aqueous anions and the potential for using these dyes in biological halide-sensing applications are discussed.

Key Words: **fluorescence; fluorescence quenching; chloride; halide ions; 6-methoxyquinoline; 6-methylquinoline; spq; halide sensing.**

It is well known that the fluorescence of many fluorophores is quenched by heavy-atom quenchers such as bromine and iodine (1). However, chloride is a less effective quencher and relatively few fluorophores are quenched by chloride. As a result, the choice of chloride-sensitive indicators for biological applications is limited. One important biological application is the diagnosis of cystic fibrosis. Few diseases can be so readily diagnosed as cystic fibrosis, which is characterized by a high chloride concentration in a patient's sweat and saliva, typically >60 mmol L^{-1}, compared to non-cystic fibrosis patients, <40 mmol L^{-1} (2). Hence, chloride-sensitive fluorescence indicators are ideally suited to measurements at these physiological concentrations and thus could provide a relatively cheap, fast, and reliable way of determining chloride concentration. At present, the Gibson–Cooke method (3) is still widely used for sweat chloride determination, although, due to the recent publication of the human genome and the subsequent advances in DNA sequencing and related technologies, there has been a shift toward genotype determination for cystic fibrosis (4).

Previously, chloride-sensitive probes were used to measure chloride transport across cell membranes (1, 5). Erythrocyte ghosts loaded with a chloride-sensitive probe and 100 mmol chloride were diluted into a solution of 66 mmol potassium sulfate. When the ghosts were diluted into the sulfate-containing buffer, the intensity of the probe increased owing to the efflux of the chloride, where the transport is due to an anion-exchange pathway, enabling physiological studies of ion transport using fluorescence quenching.

The limited use of fluorescence quenching in physiological halide determination is probably not just a consequence of the poor chloride sensitivity afforded by current dyes, but probably involves the realization that many other parameters, such as biological tissue and fluid autofluorescence (which complicates or even masks the transduction element's fluorescence kinetics), scattered light, and the quenching of the transduction element by interfering ions, are important. In this paper, three new halide-sensitive probes and three previously reported by the authors are discussed and compared along with their interferences (Fig. 1). Scattering of the excitation light is also discussed as are possibilities to alleviate biological autofluorescence.

The quenching of fluorescence was first described by Stokes as early as 1869 when he observed that the fluorescence of quinine in dilute sulfuric acid was reduced after the addition of hydrochloric acid, i.e., chloride ions. The process that he observed is now com-

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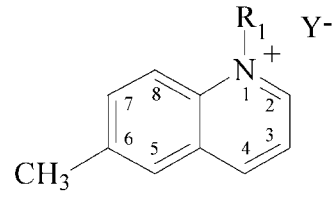
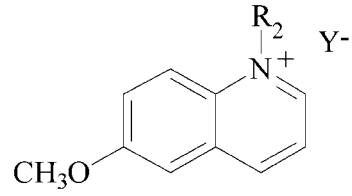


Dye 1: $R_1 = CH_3$ $Y^- = Br^-$
Dye 2: $R_1 = CH_3$ $Y^- = I^-$
Dye 3: $R_1 = CH_2CH_2CH_2OH$ $Y^- = Br^-$



Dye 4: $R_2 = CH_3$ $Y^- = Br^-$
Dye 5: $R_2 = CH_3$ $Y^- = I^-$
Dye 6: $R_2 = CH_2CH_2CH_2OH$ $Y^- = Br^-$

FIG. 1. Fluorescent dyes 1–6 and the numbering system of quinoline. The synthesis of dyes 4–6 has been described by the authors previously (9).

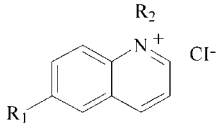
monly referred to as dynamic or collisional fluorescence quenching and is known to follow Stern–Volmer kinetics (1).

$$\frac{F'}{F} = \frac{\tau'}{\tau} = 1 + K_q\tau'[Q] = 1 + K_{SV}[Q] \qquad [1]$$

Here, F', τ' and F,τ are the intensities and lifetimes in the absence and presence of quencher, Q, respectively. K_{SV} is the Stern–Volmer constant, the magnitude of which depicts the halide concentration range detectable, and K_q is the bimolecular quenching constant.

It was perhaps using the hint that quinine, which contains a quinoline ring, was sensitive to chloride that many quinoline analogues have subsequently been made (6–8). Krapf *et al.* (7) have studied the structure–activity relationships for 6-methoxy- and 6-methylquinoline and show that high chloride sensitivity can be assigned to those probes containing the quinoline backbone substituted with electron-donating groups such as methyl and methoxy. They state that the length of the sulfoalkyl chain is relatively unimportant, although there are some changes in the K_{SV} values (Fig. 2, structures B and C), as is the position of a substituent on the quinoline backbone. Methyl or methoxy group substitution generally increases halide sensitivity, independent of the ring position substituted, although substitution of ring position 6 yielded the highest chloride sensitivity. Recently, Geddes *et al.* (8) have reported the effect of carboxylic acid chain length on the magnitude of K_{SV} values for probes based on the 6-methoxyquinoline nucleus, where functionalization occurred on the nitrogen heteroatom, i.e., ring position 1. They show that the chain length has a noticeable effect on halide sensitivity (Fig. 2, structures D, E, and F).

In this paper we have taken on-board previous findings (7, 8) and synthesized three fluorescent probes based on the 6-methylquinoline nucleus (Fig. 1, dyes 1–3) in an attempt to improve chloride sensitivity. The synthesis of the corresponding 6-methoxyquinoline dyes (dyes 4–6) has been described by the authors previously (9). By subsequently realizing and reducing the chain length of the substituent attached to the



Structure	R_1	R_2	Cl	IUPAC name	*Ksv* (M^{-1}) I⁻	Br⁻	Cl⁻	Reference
A	$-CH_3$	$-(CH_2)_3SO_3^-$	-	6-Methyl-*N*-(3-sulfopropyl) quinolinium	171	123	83	(7)
B	$-OCH_3$	$-(CH_2)_3SO_3^-$	-	6-Methoxy-*N*-(3-sulfopropyl) quinolinium[a]	276	175	118	(7)
C	$-OCH_3$	$-(CH_2)_4SO_3^-$	-	6-Methoxy-*N*-(4-sulfobutyl) quinolinium	233	154	78	(7)
D	$-OCH_3$	$-(CH_2)_7COOH$	Br^-	6-Methoxy-*N*-(8-octanoic acid) quinolinium bromide[b]	634	225	52	(8)
E	$-OCH_3$	$-(CH_2)_{10}COOH$	Br^-	6-Methoxy-*N*-(11-undecanoic acid) quinolinium bromide[b]	480	150	34	(8)
F	$-OCH_3$	$-(CH_2)_{14}COOH$	Br^-	6-Methoxy-*N*-(15-pentadecanoic acid) quinolinium bromide[b]	398	127	34	(8)

FIG. 2. Structural formulae and Stern–Volmer constants for some halide-sensitive dyes. [a]Dye B is often referred to as SPQ in the research literature. [b]K_{SV} values for dyes D, E, and F are quoted in units of mol^{-1} dm^3 in Ref. (8). CI, dye counterion. "—" indicates no dye counterion as the dye is a zwitterionic inner salt.

quinolinium backbone at ring position 1 and studying counterion quenching effects, we have successfully identified new water-soluble chloride-sensitive dyes with improved sensitivity.

EXPERIMENTAL PROCEDURES

Materials

All reagents were purchased from Aldrich Chemical Co. and were used as received. The purity of 6-methylquinoline was verified using thin-layer chromatography.

Dye Solutions

Dye solutions were made from doubly distilled deionized water with typical concentrations of 5×10^{-6} mol dm^{-3}. Buffers were not used as some have been shown to dynamically quench the fluorescence of molecules based on the quinoline nucleus (1). In accordance with previous findings, degassing samples was deemed unnecessary as oxygen shows negligible quenching of quinolinium fluorescence (10).

Instrumentation

^{1}H NMR spectra were recorded on a Jeol GSX400 (400 MHz). IR spectra were recorded on a Nicolet 5DXC FTIR spectrometer and absorption spectra on a Perkin–Elmer UV–VIS spectrometer (Lambda 2). Corrected fluorescence emission spectra were recorded on a Jobin Yvon SPEX (Fluoromax 2). Elemental analysis and positive-ion electrospray mass spectrometry were carried out by Crosfield Ltd.

Steady-State Stern–Volmer Analysis

For all experiments, the fluorescence was collected from the sample at 90° with respect to the excitation light. The samples were excited at 350 nm and the fluorescence intensity measured at 443 nm was integrated for 5 s until the standard error was better than 0.25%. Steady-state Stern–Volmer analysis of the dyes was carried out at 20°C, pH 7, using halide concentrations in the range $1–10^{-4}$ mol dm^{-3}. Subsequently, the Stern–Volmer constants were calculated using data collected in the linear halide concentration range 0.1–0.01 mol dm^{-3}, using the Microsoft Excel least-squares linear regression analysis program, where regression coefficients were typically in unity, i.e., $R^2 = 1$. All samples were measured in $4 \times 1 \times 1$-cm plastic cuvettes (Hughes and Hughes).

Synthesis Procedures

The nitrogen heteroatom of 6-methylquinoline was quaternized with methyl bromide, methyl iodide (iodomethane), and 3-bromo-1-propanol to produce dyes 1–3, respectively, which was based on the procedure previously described by Geddes *et al.* (8). This procedure has several advantages in that both the products and starting materials can be easily separated as they are relatively polar and hence water soluble and relatively nonpolar, respectively. This also allows for simple and quick bench-top product purity checks using thin-layer chromatography. In fear of halide contamination it was deemed necessary to use the low-yield synthetic routes for dyes 1 and 2 described here rather than the ion-exchange route between the dyes. ^{1}H NMR, FTIR, mass spectrometry, and elemental analysis are all consistent with the proposed products.

TABLE 1

Absorption, λ_{abs}, and Emission, λ_{em}, Wavelength Maxima (nm) for Dyes 1–3 in Various Solvents at 20°C

	Dye 1		Dye 2		Dye 3	
Solvent	λ_{abs} (nm)	λ_{em} (nm)	λ_{abs} (nm)	λ_{em} (nm)	λ_{abs} (nm)	λ_{em} (nm)
H_2O, pH 7	320	420	320	420	320	422
Methanol	320	433	320	431	320	434
Propanol	321	431	321	432	321	433
Butanol	321	430	321	431	321	432

Note. The excitation wavelength was 320 nm. λ_{abs} and λ_{em} for dyes 4–6 have been reported previously by the authors (9).

Synthesis of Dye 1

6-Methylquinoline (9.98 g, 69.7 mmol) and 60 ml (120 mmol) methyl bromide (2 N in diethyl ether) were heated under reflux at 55°C, under the exclusion of moisture for 4 h. After cooling and the addition of 75 ml diethyl ether, the mixture was stirred for 1 h and then the ether decanted. Continual washings of the product with diethyl ether gave 4.54 g (27.35%) white product.

Synthesis of Dye 2

6-Methylquinoline (9.98 g, 69.7 mmol) and methyl iodide (9.95 g, 70.1 mmol) were heated under reflux at 75°C for 5 h. After cooling and the addition of 50 ml diethyl ether, the mixture was stirred for 1 h and then the ether decanted. Continual washings of the product with diethyl ether gave 17.52 g (88.17%) yellow product.

Synthesis of Dye 3

6-Methylquinoline (5.06 g, 35.3 mmol) and 3-bromo-1-propanol (4.82 g, 34.7 mmol) were heated under reflux at 85°C for 2 1/2 h. After cooling and the addition of 30 ml acetone, the mixture was stirred for 1 h and then the acetone decanted. Continual washings of

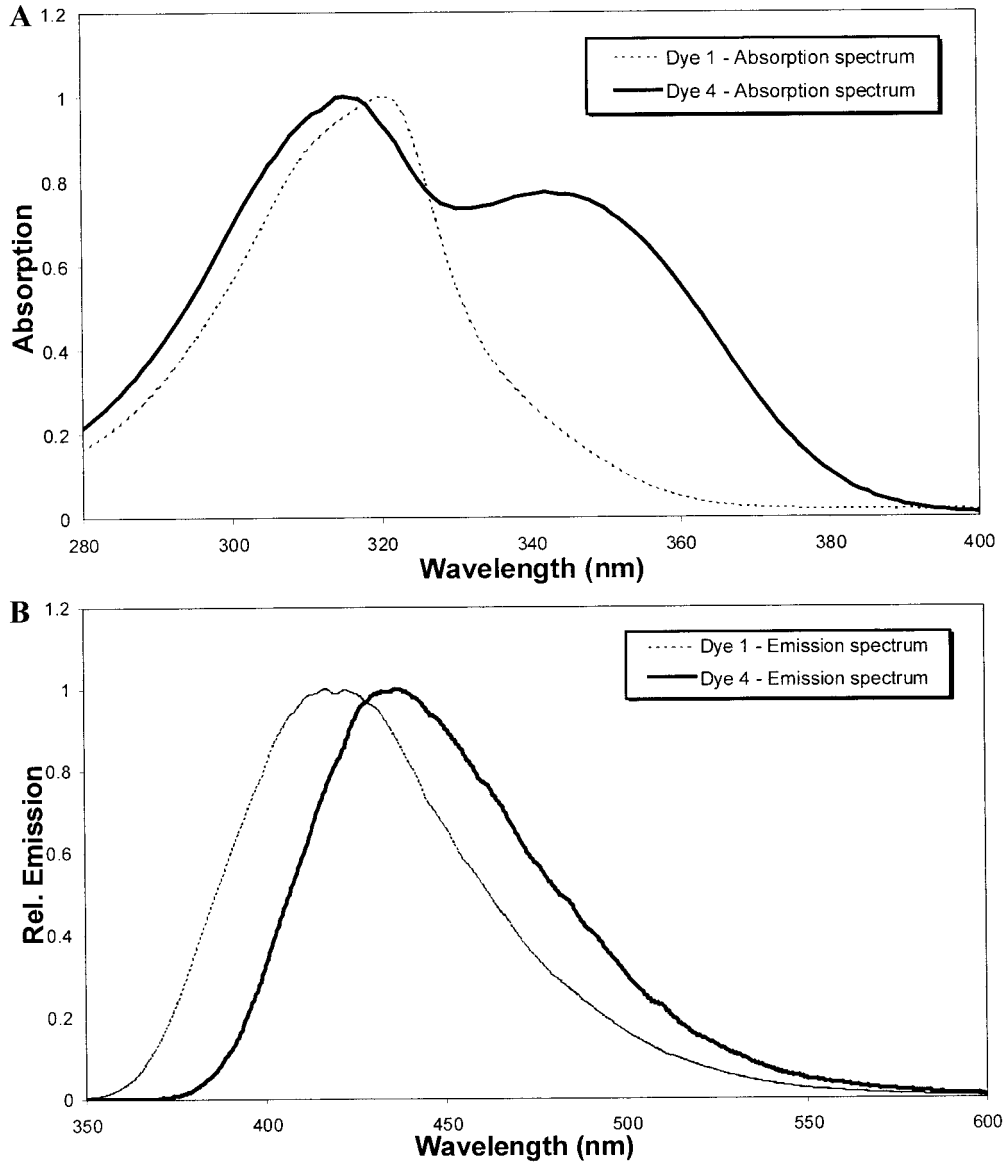


FIG. 3. (A) Absorption and (B) emission spectra of dyes 1 and 4 (H_2O, pH 7, 20°C). The excitation wavelengths for dyes 1 and 4 were 320 and 317 nm, respectively.

the product with acetone gave 5.91 g (60.40%) white product.

RESULTS AND DISCUSSION

The absorption and emission wavelength maxima for dyes 1–3 in various solvents are shown in Table 1. The emission spectra are typically broad with $\lambda_{em} \approx 420–434$ nm. They are similar to the corresponding 6-methoxyquinoline dyes (dyes 4–6), reported previously by the authors (9), lying in the range 430–444 nm and also to the emission spectrum of protonated 6-methoxyquinoline, λ_{em} max ≈ 445 nm (11). Figure 3 shows the absorption and emission spectra for dyes 1 and 4. While the emission spectra are similar, the absorption spectra for both the methyl (dyes 1–3) and methoxy (dyes 4–6) quinoline dyes are somewhat different. An interesting feature of all these dyes is the large Stokes shift in the fluorescence emission spectrum with respect to the excitation, ≈100 nm, indicating a significant difference in the energy gap between the ground and first excited singlet state of the dyes, i.e., in absorption and emission. In practice, the ease of discrimination between these two states allows for the use of "cut-off filters" rather than interference filters with only very little loss in excitation and emission energy, which is often an important consideration in immobilized dye type sensors operating at low signal/noise ratios.

TABLE 2

Steady-State Stern–Volmer Constants,[a] K_{SV} (mol^{-1} dm^3), for dyes 1–6 in H_2O, pH 7, Measured at 20°C

Dye	K_{SV} I^-	K_{SV} Br^-	K_{SV} Cl^-
1	645	421	255
2	370	266	162
3	526	346	219
4	307	278	113
5	294	282	107
6	357	241	132

[a] Stern–Volmer constants have been corrected to three significant figures.

Solution Stern–Volmer constants for dyes 1–6 quenched by aqueous halide ions at pH 7 are presented in Table 2. As expected, the halide sensitivity for all the dyes is in the order $I^- > Br^- > Cl^-$. K_{SV} values for the 6-methylquinoline dyes (dyes 1–3) are typically greater than those for the corresponding 6-methoxyquinoline dyes (dyes 4–6). However, the opposite has been observed for similar sulfoalkyl quinolinium dyes (7) (Fig. 2, structures A and B). We can also see from Table 2 that the presence of a long alkyl chain for the 6-methylquinoline dyes typically reduces the K_{SV} values, i.e., a comparison of dyes 1 and 3. The opposite, however, is observed for the 6-methoxyquinoline dyes, dyes 4 and 6, respectively.

It can be seen from Table 2 that dye counterions have a significant effect on the value of K_{SV}. Recently, Geddes *et al.* (8) reported the steric hindrance effect of counterion *size* on the magnitude of dye K_{SV}. Replacing the counterion of dye D in Fig. 2, from a bromide to a tetraphenylboron ion, had the effect of reducing the K_{SV} to 218, 70, and 20 mol^{-1} dm^3 for I^-, Br^-, and Cl^-, respectively (8). Here, it is unlikely that the size of the counterion is the dominant factor influencing the magnitude of K_{SV}. Bromine and iodine are well known to be heavy-atom quenchers (1, 12), whereby spin-orbit coupling between excited dye and halide increases the triplet yield of the dye via intersystem crossing from the excited singlet state. The relatively long-lived triplet state is probably then quenched (e.g., O_2 quenched) and hence no phosphorescence is observed. Since this effect depends on the mass of the heavy atom (12), and given $I^- > Br^-$, the reduction in the magnitude of K_{SV}, which also follows this trend, is attributed to a heavy-atom quenching effect by the dye counterions. The introduction of additional aqueous halide to dye solutions, i.e., to produce Stern–Volmer plots, results in collisional "dynamic" fluorescence quenching since the dye/counterion pair is already present in normal solutions of these dyes. It is worth noting at this point that one would therefore expect the chloride counterion analogue of dye 1 to give an even greater halide K_{SV}, although this has not been synthesized and reported as of yet. Further, the K_{SV} values in this paper were recorded at a dye concentration of 5×10^{-6} mol dm^{-3} and slightly increasing the dye concentration was seen to have little effect on K_{SV} values, suggesting that the halide counterions do not further act as dynamic quenchers (1). Also, it is well known that a combination of both dynamic and static quenching leads to a modified form of the Stern–Volmer equation (Eq. [2]), which is second order in [*Q*] and typically shows an upward curvature. Clearly, this quenching mechanism is not evident here as a linear halide dependence is observed (Fig. 4).

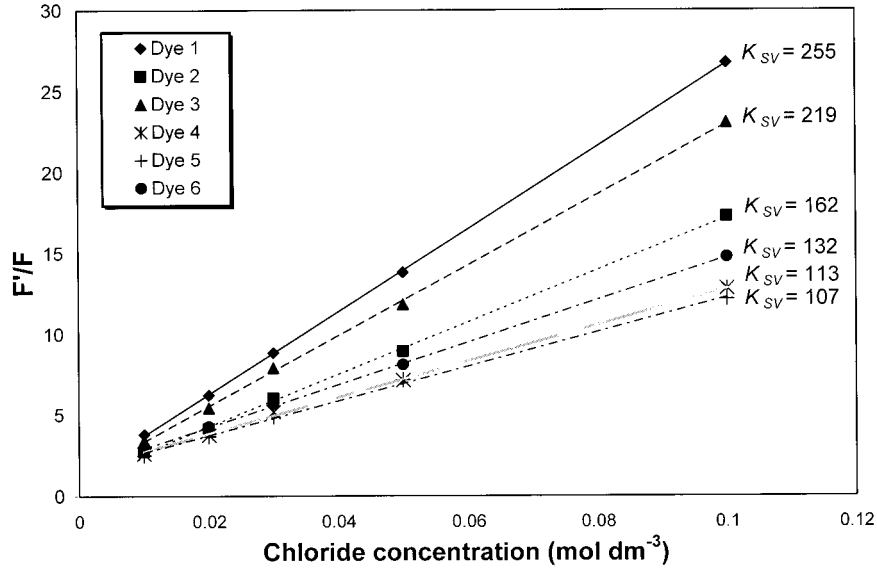


FIG. 4. Stern–Volmer plots for dyes 1–6 quenched by chloride ions, H_2O, pH 7, 20°C. K_{SV} units, mol^{-1} dm^3.

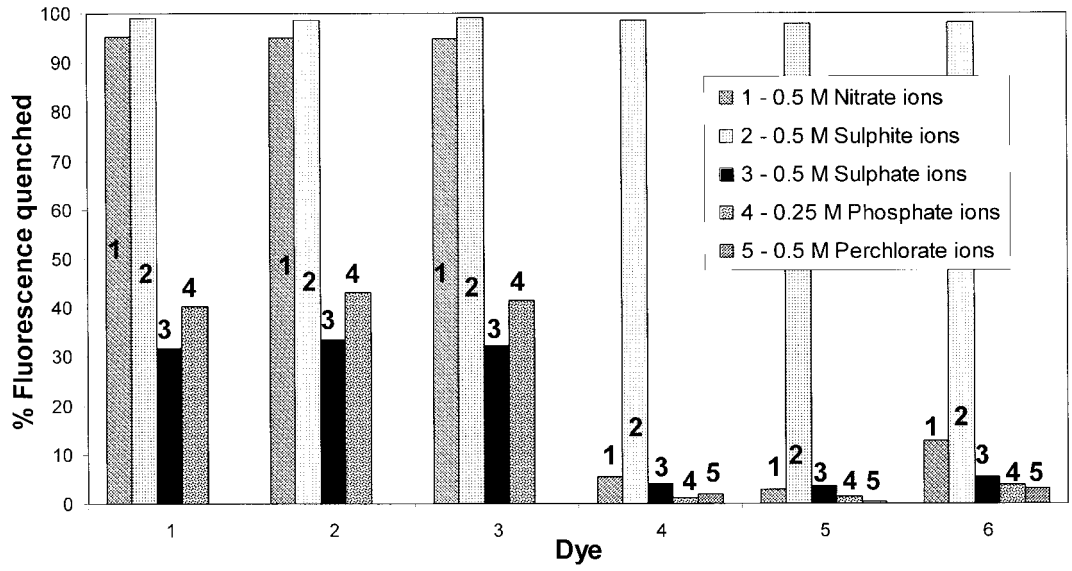


FIG. 5. Percentage fluorescence quenched of 10^{-6} mol dm^{-3} dye 1–6 solutions by various aqueous anions. Dyes 1–3 were not quenched by perchlorate ions.

$$\frac{F'}{F} = (1 + K_{SV}[Q])(1 + K_{static}[Q]) \qquad [2]$$

Here, K_{static} is the static Stern–Volmer quenching constant (1).

Another type of static quenching that is often observed at high quencher concentrations is due to the existence of an increasing number of quencher–fluorophore pairs in which the quencher is close enough to the fluorophore upon excitation to instantly quench its excited state, namely the *sphere of action* static quenching model (1).

$$\frac{F'}{F} = (1 + K_{SV}[Q])e^{V[Q]} \qquad [3]$$

At high halide concentrations, typically >0.2 mol dm^{-3}, negative deviations from Stern–Volmer behavior are observed. Attempts to fit the data to models such as the sphere of action static quenching model, which assumes a diffusionless and finite volume, V, within which quenching occurs, and which typically also shows upward curvature, were unsuccessful. However, for halide concentrations of biological interest, i.e., <0.1 mol dm^{-3}, Stern–Volmer plots are linear and reproducible which would allow the use of these dyes in biological halide-sensing applications. Further, it is likely that fluorescence lifetime data may provide additional information on the quenching mechanism. Further studies are underway.

A striking feature of both Table 2 and Fig. 4 is the magnitude of the Stern–Volmer constants and hence chloride sensitivity of these dyes. Krapf *et al.* (7) have previously reported chloride K_{SV} values in the range 3–118 M^{-1} for their quinolinium dyes and hence it can be seen that dye 1, which has a Cl^- K_{SV} of 255 mol^{-1} dm^3, is one of the most sensitive reported, for the quinolinium type dyes. It should be noted that, to date, very few molecules have larger aqueous chloride K_{SV} values than dye 1. One notable exception is lucigenin (13), Cl^- K_{SV} = 390 M^{-1}, although this dye is based on the acridine nucleus.

To ascertain dye selectivity, interferences were studied by using 0.25 and 0.5 mol dm^{-3} standard solutions of various anions (Fig. 5). At these interference concentrations, sulfite was found to be an efficient quencher of all the dyes while phosphate was found to be a modest quencher for dyes 1–3, and, surprisingly, 0.5 mol dm^{-3} nitrate was also found to be an efficient quencher for dyes 1–3. In contrast, for dyes D, E, F in Fig. 2, no interferences were reported using 0.5 mol dm^{-3} nitrate, sulfate, or phosphate ions. In sensing applications, anion interferences can be taken into account and hence are not necessarily a problem, by making use of a modified form of the Stern–Volmer equation, first proposed by Wolfbeis and Urbano in 1983 (14).

Both bromide and iodide determinations, as well as chloride determination, are deemed equally important in human physiology. The human body contains on average 14 mg of iodine, concentrated mostly in the thyroid gland. Iodine-containing hormones (thyroxine, tri-iodothyronine) strongly influence a range of biological reactions. Typically, iodine species such as I^- and IO_3^- are absorbed from food and reduced to iodide (or remain as iodide) in the gastrointestinal tract, where the absorbed iodide is considered as the main source of iodide for the synthesis of these hormones. About 1 mg

of iodine per week is needed to ensure the normal synthesis of these thyroid hormones (15). Typical I^- and total iodine (organic bound + free iodide) concentrations in serum for healthy patients are in the range 2 to 4 and $\approx$80 μg L^{-1}, respectively (16). The determination of the total amount of iodine in biological materials has become a routine procedure (17), whereas the determination of free inorganic I^- in biological samples in the presence of iodinated organic compounds still remains a problem, although there is a considerable amount of interest from the medical point of view. While the physiological significance of chloride and iodide is well known, bromide is still considered as either a nonessential trace element or a trace element with an unknown function (18). However, much more is known about the toxicity of bromide (19) and hence its determination is deemed equally as important. Many authors have reported mean serum bromide levels to be in the range 2 to 8 mg L^{-1}, using techniques such as colorimetry and X-ray fluorescence spectroscopy (20). It is perhaps somewhat surprising that chloride determination using fluorescence quenching has attracted slightly more attention than both iodide and bromide, although this may be due to the fact that physiological chloride levels are typically higher than bromide or iodide and hence easier to determine. This may change given the recent reports of improved dye halide sensitivity/selectivity and models capable of describing multiple or distributions of quenchers (1, 21), which could therefore be applied to complex conditions such as organic bound and free inorganic iodide.

CONCLUSIONS

The synthesis, sensitivity, and selectivity of three dyes based on the 6-methylquinoline nucleus have been described. These water-soluble probes offer improvements in chloride sensitivity over some previous quinolinium dyes. A comparison with similar dyes based on the 6-methoxy quinoline nucleus provides an additional insight into the structure–activity relationships of quinolinium analogues. However, for halide-sensing applications, one disadvantage of these types of dyes is their requirement for UV excitation, typically $<$350 nm. At this wavelength one would have to tune sensor devices to accommodate the optical properties of water and tissues. In general, the autofluorescence from tissues or any biological fluid is lower for longer excitation wavelengths (1). At longer wavelengths one also avoids the absorption of hemoglobin and melanin, but unfortunately very few halide-sensitive dyes absorb in this region, 600–1000 nm, often referred to as the therapeutic range (1). One possible solution may be to use multiphoton excitation (21–23). As well as being able to use current dyes, near-infrared multiphoton excitation has the added attraction of reduced sample degradation and reduced scattering of the excitation light, due to the Rayleigh λ^{-4} dependence (22). To date, no reports of multiphoton Stern–Volmer kinetics with respect to halide sensing have been published.

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