Fluorescent Probes and Labels for Biomedical Applications

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Fluorescence probes and labels have become indispensable tools for clinical diagnostics, high-throughput screening, and other biomedical applications. We have developed several classes of new squaraine-based red and near-infrared (NIR) probes and labels (SETA and Square series), naphthalimide-based fluorescence lifetime dyes (SeTau series), and cyanine- and squaraine-based quenchers (SQ series). This report discusses the spectral and photophysical properties of these new markers. In particular, the red and NIR dyes of the SETA and Square series are extremely bright, with photostabilities that are unmatched by any other dyes in the same spectral region.

Key words: **biomedical probes and labels; quenchers; lifetime labels; pH-sensitive dyes; biological imaging**

Introduction

Fluorescent but also nonfluorescent dyes (quenchers) are used for a wide range of biomedical applications. Whereas fluorescent labels are used for covalent attachment to biological species, fluorescent probes undergo noncovalent interactions with a biomolecule or the analyte of interest. Despite the many commercially available fluorescent dyes, they still suffer from shortcomings, such as low fluorescence quantum yields (QYs) in aqueous media and insufficient photostability. Also, there is a need for not only brightly fluorescent dyes but also truly dark quenchers for energy transfer measurements that exhibit no residual fluorescence. In particular, in the red and near-infrared (NIR) region, the lack of bright, photostable markers for imaging applications is still pronounced.

Recently we reported the synthesis, spectral properties, and biomedical applications of squaraines and ring-substituted squaraines (Square and SETA dyes).[1–6] Here we describe new probes and labels and compare them with other commercially available markers. Meanwhile we introduce the new SeTau and SQ series of dyes, which are represented by fused aromatic compounds and cyanine dyes, respectively. These are a series of extremely bright and photostable fluorescence dyes and dark quenchers for use in biological and pharmaceutical research, clinical diagnostics, and high-throughput screening. These materials include

- reactive red and NIR fluorescent labels of the Square and SETA series for covalent attachment to biomolecules, such as proteins, amino acids, peptides, oligonucleotides, DNA, RNA, lipids, and drugs;
- fluorescent probes for noncovalent labeling of proteins, lipids, and cells;
- pH-sensitive probes and labels;
- fluorescence lifetime (FLT) probes and labels of SeTau series for FLT- and fluorescence polarization–based applications; and
- dark quenchers of the SQ series for fluorescence resonance energy transfer (FRET) applications.

Experimental Methods

Chemicals

Square, SETA, SeTau, and SQ dyes (K1, K7, and K8 series) and their *N*-hydroxysuccinimide (NHS) esters were from SETA BioMedicals (Urbana, IL; http://www.setabiomedicals.com) and used as is. Bovine serum albumin (BSA; essentially fatty acid free),

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human serum albumin (HSA; fraction V) and human immunoglobulin G (IgG) were purchased from Sigma (St. Louis, MO); all other chemicals and solvents were from Merck (Merck & Co., Inc., Whitehouse Station, NJ; Merck KGaA, Darmstadt, Germany). All chemicals were used without further purification.

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 dimethylformamide was prepared; 5 mg of BSA or 3 mg of IgG protein was dissolved in 1 mL of a 50 mM bicarbonate buffer (pH 9.0), and various amounts of dye from the stock solution were added and the mixture was allowed to stir for 2 h at room temperature (RT). Unconjugated dye was separated from the labeled protein by using gel permeation chromatography with Sephadex G25 for BSA conjugates or Sephadex G50 for IgG (0.5 × 20–cm column) and a 67 mM phosphate buffer (PB) solution of pH 7.4 as the eluent. A series of labeling reactions as described above were set up to obtain different dye-to-protein (D/P) ratios. The D/P values were calculated according to the method of Mujumdar *et al.*[7]

Absorption Spectra and Extinction Coefficients

Absorption spectra were measured at RT on a Perkin-Elmer Lambda 35 ultraviolet/visible spectrophotometer (Shelton, CT). For determination of the extinction coefficients, each dye (7–10 mg) was dissolved in 50 mL of PB, pH 7.4. The stock solution was diluted (1:2000) and the absorbance (A) was measured in a 5-cm standard quartz cell. All the dye concentrations were between 0.1 and 0.2 μM. The extinction coefficients were calculated according to the Lambert–Beer law.

Fluorescence Spectra and QYs

Fluorescence spectra and QYs for the dyes, dye–BSA, and dye–IgG conjugates were measured in PB at RT on a Varian Cary Eclipse spectrofluorometer (Varian Australia Pty Ltd., Sydney, Australia) in a standard 1-cm quartz cell. The spectra were corrected. All concentrations of the fluorophores were chosen to be between 0.1 and 0.2 μM.

For the determination of QYs, the integrated relative intensities of the dyes or dye–protein conjugates were measured against Cy5 as the reference. All absorbances at excitation wavelength (λ_{exc}) were in the range of 0.12–0.18 (when measured in a 5-cm cell). The fluorescence spectra of the solutions were measured and the absolute QYs were determined relative to Cy5 (QY 27%[7]) by a previously described method.[8]

The QY of each sample was independently measured three to four times, and the average value was calculated. The QYs of the dye–BSA conjugates were measured for various D/P ratios, and then the QYs for D/P = 1 were determined by a nonlinear interpolation.

Polarization

Excitation polarization spectra were measured at RT in PB on a Varian Cary Eclipse spectrofluorometer, using standard film polarizers for excitation and emission, in a standard 1-cm quartz cell. All dye concentrations were between 0.1 and 0.2 μM. Polarization values (P) were measured and calculated by a previously described method.[8]

FLT

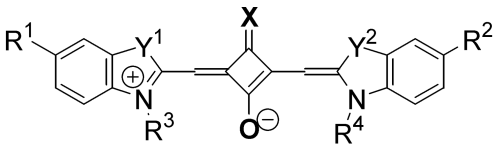
FLT measurements were acquired using Chronos—a laser-diode–based frequency domain instrument (ISS, Champaign, IL). The dye concentrations in PB, pH 7.4, were in the range of 0.1–1.0 μM. We analyzed data with Vinci Multidimensional Fluorescence Spectroscopy—a comprehensive software package for the analysis of time-resolved data from ISS.

Photostability

Photostability measurements were performed in aqueous solutions of the Cy5, Alexa 647, Square, and SETA dyes, and their BSA conjugates were prepared. The optical density at the long-wavelength maximum was controlled to be between 0.09 and 0.1. Measurements were carried out in standard 1-cm cells. The solutions were placed approximately 30 cm from a 500-W incandescent lamp and irradiated with occasional stirring. The absorption and emission spectra of the solutions were measured before irradiation and during light exposure. The relative photostabilities were calculated as the ratio between (1) the measured absorbances at the long-wavelength maximum before and after exposure (A/A_0) and (2) relative fluorescence intensities before and after exposure (I/I_0), and the corresponding plots were generated.

Staining of Cells

Staining of cells with noncovalent probes was performed by adding the dye ethanolic solution to an aqueous suspension of the cells and stirring at RT for 15 min. Because the unbound probe was nonfluorescent in aqueous solutions, rinsing steps were not critical. Covalent labeling with dyes was carried out by treatment of an aqueous suspension (PB, pH 7.4) of the cells with a dimethylformamide solution of NHS ester



Y^1, Y^2 = O, S, CMe_2, $C(Me)(CH_2)_5COOH$;
R^1, R^2 = H, Me, SO_3H; R^3, R^4 = H, Me, $(CH_2)_5COOH$, $(CH_2)_4SO_3H$

SCHEME 1. General formula of Square and Seta dyes.

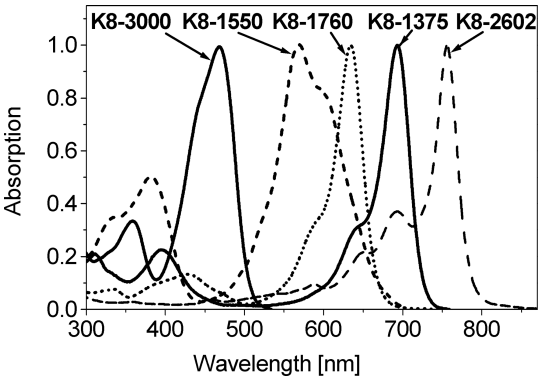


FIGURE 1. Absorption spectra of representative squaraine markers in phosphate buffer pH 7.4.

at RT for 30 min. Subsequently, the stained cells were separated from free, unbound dye by centrifugation at 600 *g* on a 310 b centrifuge (Mechanika Precyzyina, Warsaw, Poland) and washed with PB.

Results and Discussion

Square and SETA probes and labels (K8 series) have been developed based on squaraine and ring-substituted squaraine dyes. In ring-substituted squaraines, the squaric acid oxygen is replaced by other heteroatoms and groups.[1,2] The general formula of these dyes is given in SCHEME 1. Water-soluble, reactive SETA labels (NHS esters and maleimides) exhibit a lesser tendency to aggregate and higher fluorescence QYs than those of Square dyes. They are perfectly suited for covalent labeling of both high- and low-molecular-weight analytes such as amino- and thiol-modified peptides, oligonucleotides, proteins, DNA, RNA, and lipids. Square dyes include amine- and thiol-reactive labels but also probes for noncovalent binding. They are available as both water-soluble and hydrophobic forms. Square dyes are typically better suited for covalent and noncovalent labeling of high-molecular-weight analytes, such as proteins, lipids, membranes, and cells.[3–6]

Spectral Range

Square and SETA markers absorb (between 300 and 800 nm) and emit (between 500 and 850 nm) over a wide spectral range. Unlike open-chain cyanine dyes of the Cy and Alexa Fluor series, the absorption spectra of the red- and NIR-emitting markers, such as K8-1260, K8-1340, K8-1343, and K8-1760, exhibit an additional absorption band with maxima around 350–450 nm and extinction coefficients of 16,000–31,000 $M^{-1}\cdot cm^{-1}$ (FIG. 1, TABLE 1). These extinction coefficients are of the same order as for some of the most common fluorophores in this spectral region, which makes these red- and NIR-emitting labels compatible not only with the red 635-nm and 670-nm diode lasers but also with the ultraviolet and blue diode lasers and light-emitting diodes at 380, 405, and 436 nm. Importantly, the QYs are independent of the excitation wavelength.

Brightness

The brightness of a dye is defined by its extinction coefficient and QY. Square and SETA dyes have high extinction coefficients (up to 280,000 $M^{-1}\cdot cm^{-1}$ for K8-1640), and protein conjugates of these dyes are extremely bright (TABLE 1). QYs (up to 60%) for the BSA conjugates of selected squaraines, such as K8-1640, K8-1643, and K8-1661, are unmatched by open-chain cyanines such as Cy5 or Alexa 647 dyes. FIGURE 2 shows the QYs of dye–BSA conjugates at different D/P ratios for K8-1643 compared with Cy5 and Alexa 647.

Photostability

Next to brightness, the photostability of probes and labels plays an important role for their practical application. The photostability of cyanine dyes absorbing and emitting in the red and NIR spectral ranges is substantially lower than that for other classes of organic dyes. Therefore, photostability is one of the most important characteristics of the long-wavelength dyes. Importantly, the red and NIR dyes of the Square and SETA series were more photostable than were Cy5 and

TABLE 1. Spectral characteristics of dyes and BSA and IgG conjugates (D/P = 1) in phosphate buffer, pH 7.4

Dye or dye conjugate	λ_{max} (Ab) [nm]	ε [$M^{-1} \cdot cm^{-1}$]	λ_{max} (Em) [nm]	QY [%]
Cy5	647	250,000	664	27[7]
Cy5–BSA	653		672	20
Cy5–IgG	651		670	29
Alexa 647	649	167,000	668	27
Alexa 647–BSA	658		674	39
Alexa 647–IgG	652		670	42
K8-1250	636	216,000	648	6
	370	23,000		
K8-1260	640	120,000	653	5
	370	12,000		
K8-1260–BSA	655		663	16
K8-1260–IgG	646		658	13
K8-1340	667	179,000	686	7
	381	31,000		
K8-1340–BSA	681		696	38
K8-1343	667	118,000	683	3
	389	28,000		
K8-1343–BSA	682		697	37
K8-1343–IgG	669		687	14
K8-1355	654		671	1
K8-1355 + 1 mg/mL BSA	688	168,000	708	62
K8-1431	630		662	0.1
K8-1431 + 1 mg/mL BSA	684	183,000	704	58
K8-1440	659		677	<0.1
K8-1440 + 1 mg/mL BSA	683	196,000	702	50
K8-1500	622		644	<0.1
K8-1500 + 1 mg/mL BSA	652	177,000	672	80
K8-1550	570	60,000	641	7
K8-1640	632	280,000	641	6
K8-1640–BSA	644		654	43
K8-1640–IgG	637		647	18
K8-1643	634	181,000	644	4
K8-1643–BSA	650		660	56
K8-1643–IgG	638		650	15
K8-1661	633	250,000	644	7
K8-1661–BSA	646		656	53
K8-1661–IgG	637		647	26
K8-1760	634	131,000	656	8
	427	18,000		
K8-1760–BSA	650		672	36
K8-1760–IgG	650		671	23
K8-1649	740	44,000	No	No
K8-1902	560	22,000	No	No
K8-2602	756	200,000	No	No
K8-2602–BSA	765		No	No

No, nonfluorescent.

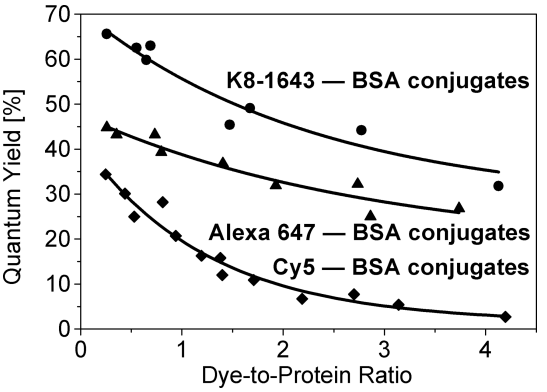
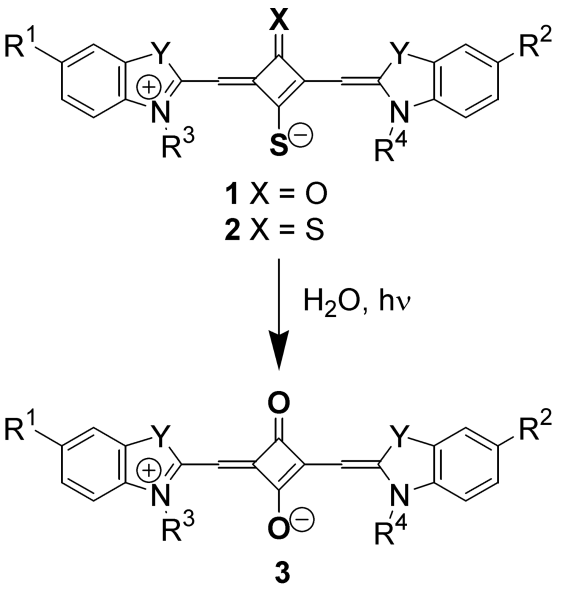


FIGURE 2. Quantum yield of dye–BSA conjugates versus dye-to-protein ratio.

Alexa 647 cyanine dyes. The relative photostabilities for selected dyes, calculated as the ratio between the measured absorbances (A/A_0) or relative fluorescence intensities (I/I_0) before and after exposure, are given in TABLE 2. Representative stability curves are shown in FIGURE 3. The improved photostability is due to the introduction of electron-withdrawing substituents X, such as dicyanomethylene, butyloxycarbonyl(cyano) methylene, butyloxycarbonyl(nitro) methylene, barbituric and thiobarbituric acids, and indandione, in the squaraine ring.

We observed that the photobleaching tendency can be reduced by substitution of squaric oxygen with a sulfur atom. Whereas conventional, open-chain cyanines and oxosquaraines, such as 3 photobleach upon exposure to light, the absorbance and emission intensity of the thiosquaraines 1 and 2 increased (SCHEME 2). FIGURE 3 shows change of fluorescence intensity of aqueous solutions of thiosquaraine label K8-1260 compared with that of Cy5 and Alexa 647. The described effect can be attributed to a photoinduced hydrolysis of the thiosquarylium C–S group whereby the thionated dyes 1 and 2 are transformed into oxosquaraine dye 3 (SCHEME 2). Because the photodecomposition of 3 is much slower than the hydrolysis rate of 1 or 2, and because 3 has a higher extinction coefficient and higher QY than those of 1 and 2, the absorption and emission intensity increases until 1 and 2 are totally transformed to 3. During this process, only a small blueshift of the absorption and emission maxima (no more than 16 nm) is observed. Neither thiosquaraine 1 nor 2 hydrolyzes in the absence of light. For practical applications, the increase of fluorescence intensity of protein-bound thiosquaraines upon light exposure is found to be even more pronounced than that for the free dyes.



$Y = S, CMe_2, C(Me)(CH_2)_5COOH;$

$R^1, R^2 = H, SO_3H; R^3, R^4 = Me, (CH_2)_5COOH, (CH_2)_4SO_3H$

SCHEME 2. Photoinduced transformation of thiosquaraines into oxosquaraines.

TABLE 2. Relative photostabilities of the selected dyes measured in 1.5 h of light irradiation

Dye	A/A_0	I/I_0
Cy5	0.50	0.46
Alexa 647	0.77	0.74
K8-1260	1.11	1.37
K8-1340	0.96	0.92
K8-1343	0.94	0.90
K8-1643	0.88	0.84
K8-1663	0.89	0.79
K8-1760	0.94	0.94

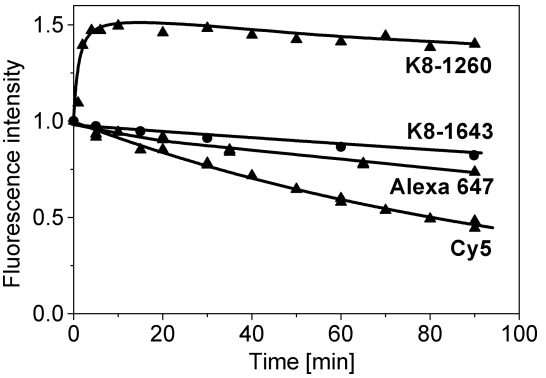


FIGURE 3. Normalized fluorescence intensity versus exposure time for free dyes. A halogen lamp (500 W) was used as the light source.

High photostability of the developed Square and SETA probes and labels is an advantage in biological imaging applications in which longer exposure times help to improve the image quality. FIGURE 4 shows the relative change in fluorescence intensity of *Saccharomyces cerevisiae* (yeast) cells stained with Alexa 647 and thiosquaraine label K8-1250 before and after 5 min of continuous irradiation during observation by an Olympus IX-71 fluorescent microscope (Olympus America Inc., Center Valley, PA) equipped with a 50% gray filter (for excitation) and a Cy5 filter set. The image segments are given as is, that is, without any processing. The cells were stained with Alexa 647 and K8-1250 NHS esters. The fluorescence image obtained with K8-1250 remains almost unchanged during the 5-min observation time, whereas Alexa 647 significantly photobleaches during this period.

Sensitivity to Microenvironment

Red and NIR probes and labels of SETA and especially the Square series show noticeable increases of fluorescence intensity and longer FLTs in the presence of large biomolecules such as proteins and lipids. Fluorescence intensity increases of up to 100 times and higher were observed. The fluorescence intensity

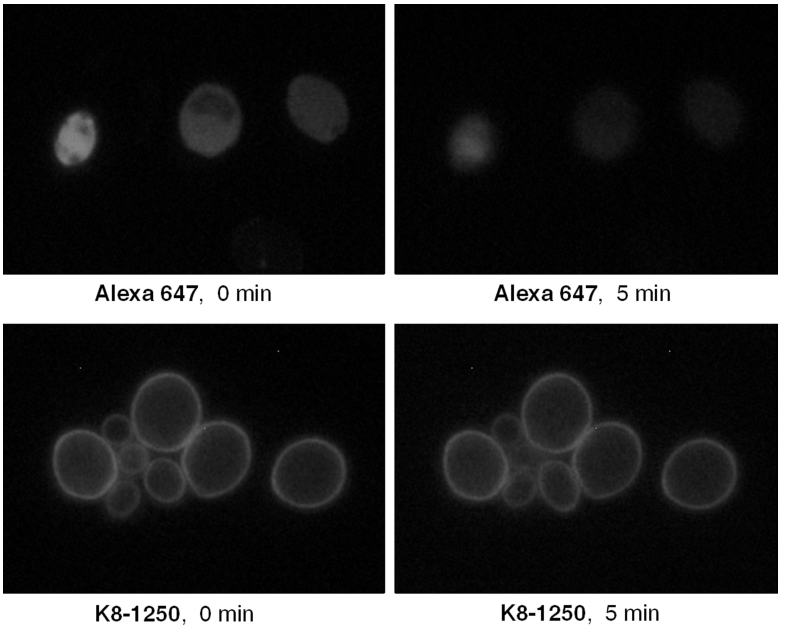


FIGURE 4. *Saccharomyces cerevisiae* (yeast) cells stained with Alexa 647 and thiosquaraine label K8-1250 before and after 5 min of observation by an Olympus IX-71 fluorescent microscope. A 50% gray filter and a **Cy5** filter set were used.

of dyes, such as K8-1355, K8-1431, K8-1440, and K8-1500, substantially increases upon binding to BSA (TABLE 1) or other biomolecules. The QYs for some of the noncovalent complexes of these dyes with BSA are as high as 80%. Therefore, these probes are perfectly suited for fluorescence-based quantification of proteins (FIG. 5). Because these dyes readily stain cells of different nature, they are also useful for *in situ* biological imaging. Furthermore, they are useful as stains for gel electrophoresis.

Because the protein binding constants of squaraines and naphthalic acid derivatives are comparable to those of some common drugs, they are used for the assessment of drug-binding constants by the dye-displacement method.[2,9–12] Thus, the HSA–dye binding constant for naphthalic derivative K7-1045 is 3.4×10^5 M^{-1}, and the number of binding sites is 1.8. The probe is nonfluorescent in water and substantially increases its fluorescence after binding to HSA molecules. K7-1045 competed with drugs such as warfarin and propranolol for HSA binding sites. As a result, titration of the warfarin–HSA and propranolol–HSA complexes with K7-1045 significantly enhanced fluorescence at approximately 520–530 nm, indicating that the dye was binding to HSA. Furthermore, back-titration of the K7-1045–HSA complex with warfarin and propranolol decreased the dye fluorescence, showing that K7-1045 was located in both warfarin and propranolol binding sites. The effectiveness of the displacement of K7-1045 by warfarin was found to be about twice as high as that for propranolol. These experiments demonstrate that K7-1045 can be used to study interaction of drugs with albumin.

pH-Sensitive Labels for Biological Applications

In recent years there has been an increased interest in the use of pH-sensitive red and NIR labels for intracellular and biomedical studies. In particular, the CypHer dye series has been used to monitor apoptosis, malignancy, cell proliferation, and receptor-mediated endocytosis.[13,14] In general, most commercially available pH-sensitive fluorescent dyes that are used for biomedical applications emit between 350 and 550 nm and lack a functional group for biolabeling.[9] On the basis of squaraines that contain a hydrogen substituent at the indolenine nitrogen, we developed and investigated several pH-sensitive long-wavelength labels of the Square and SETA series shown in SCHEME 3. Substituent X is oxygen, dicyanomethylene, butyloxycarbonyl(cyano) methylene, butyloxycarbonyl(nitro) methylene, barbituric and thiobarbituric acids, and indandione. Their absorption and emission spectra, extinction coefficients, QYs, FLTs, and polarization properties were measured in aqueous media, free in solution and after binding to BSA and IgG.

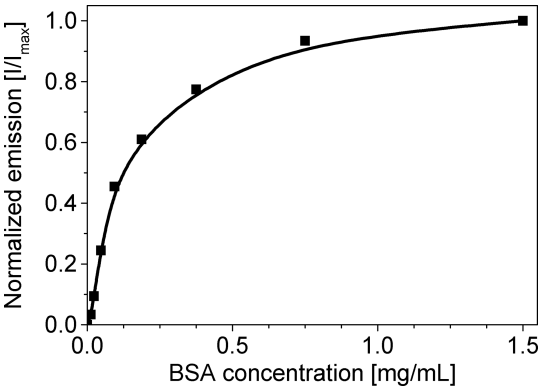


FIGURE 5. Normalized emission of K8-1500 in BSA solutions versus BSA concentration.

TABLE 3. Characteristics of pH-sensitive labels in water (protonated/deprotonated forms)

Label	pKa	pH Range	λ_{max} (Ab) [nm]	λ_{max} (Fl) [nm]
K8-1405	7.17	5.2–9.0	653/535	671/663
K8-1365	8.86	6.5–11.0	672/537	694/N/A
K8-1765	9.37	7.3–11.1	641/514	668/N/A
K8-1375	9.56	8.8–11.5	693/557	714/N/A
K8-1775	9.92	8.2–11.6	662/539	684/N/A
K8-1665	10.29	8.4–11.8	640/519	656/N/A

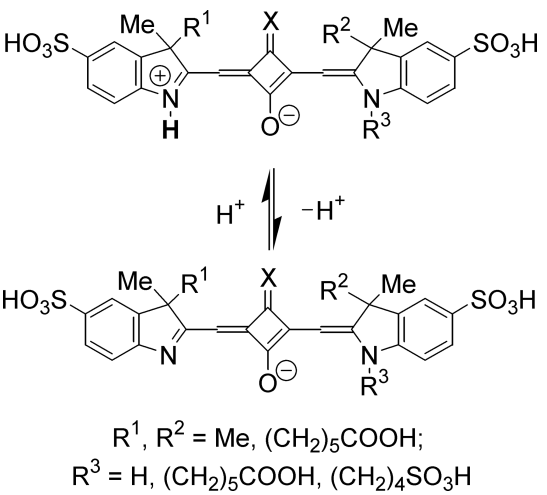
N/A, nonfluorescent.

Protonated forms of the free dyes absorb between 640 and 693 nm (long-wavelength absorption maximum) with extinction coefficients (ε) 87,000–188,000 $M^{-1}\cdot cm^{-1}$ and fluoresce between 656 and 714 nm (TABLES 3 and 4). Excitation of these labels with a 635- or 670-nm diode laser results in improved signal-to-noise ratios because of reduced background from the biological sample in the red and NIR regions. The additional short-wavelength absorption band ($\varepsilon = 16{,}000–30{,}000\ M^{-1}\cdot cm^{-1}$) in the spectra of these dyes allows excitation with a 380, 405, or 436-nm diode laser. These new dyes exhibit adequate QYs in aqueous media and when covalently bound to protein.

Because of reversible deprotonation in basic environment (SCHEME 3), the long-wavelength absorption band of these dyes at 640–693 nm decreases and a new absorption band at 514–557 nm appears whereby the fluorescence of almost all the investigated dyes is totally quenched. An exception is K8-1405, where both the protonated and deprotonated forms are fluorescent (TABLES 3 and 4). Compared with the protonated form, the fluorescence emission maximum of the deprotonated form is blueshifted by only 8 nm. As a result, the Stokes shift of the deprotonated form of K8-1405 is extremely large for this polymethine dye—more than 3600 cm^{-1}, which is one of the largest Stokes shifts that has been observed in a cyanine-based dye.

TABLE 4. Spectral characteristics of pH-sensitive dyes and their conjugates with BSA and IgG in phosphate buffer (D/P = 1)

Dye	pH	λ_{max} (Ab) [nm]	ε [$M^{-1}\cdot cm^{-1}$]	λ_{max} (Em) [nm]	QY [%]
K8-1365	6.0	672	93,000	694	15
		389	30,000		
K8-1365–BSA	6.0	693		712	8
K8-1365–IgG	6.0	684		703	7
K8-1375	6.0	693	113,000	714	14
		395	26,000		
K8-1375–BSA	6.0	711		726	14
K8-1405	5.6	653	135,000	671	16
K8-1405	9.0	535	48,000	663	9
K8-1405–BSA	5.6	670		685	5
K8-1405–IgG	5.6	664		676	1
K8-1665	7.4	640	188,000	656	33
K8-1665–BSA	7.4	655		670	16
K8-1665–IgG	7.4	646		664	21
K8-1765	7.4	641	87,000	670	21
		425	16,000		
K8-1765–BSA	7.4	660		689	10
K8-1775	7.4	662	87,000	684	17
K8-1775–BSA	7.4	677		696	19



SCHEME 3. Prototropic equilibrium in squaraine dye molecule.

The pKa for each dye was calculated using both absorption and emission spectral data. Data in TABLE 3 demonstrate that by varying the substituents R^1–R^3 and X in the indolenine and squaric moieties, one can obtain a series of pH-sensitive markers with tunable pKa values in the range between 7.17 and 10.29. These dyes can be used as highly sensitive pH indicators

TABLE 5. Fluorescence lifetimes (τ) of dyes and their BSA and IgG conjugates in phosphate buffer pH 7.4

Dye	D/P	λ_{max} (Ab) [nm]	λ_{max} (Em) [nm]	τ_1 [ns]	f_1	τ_2 [ns]	f_2	τ_{mean} [ns]
Cy5	—[a]	647	664	1.00	1.00	—[b]	—[b]	1.00
Cy5–BSA	0.9	653	672	0.28	0.10	1.88	0.90	1.72
Cy5–IgG	1.9	651	670	0.28	0.23	1.47	0.77	1.20
Alexa 647	—	649	668	1.17	1.00	—	—	1.17
Alexa 647–BSA	0.9	658	674	0.33	0.07	1.91	0.93	1.80
Alexa 647–IgG	1.8	652	670	0.31	0.20	1.29	0.80	1.09
K8-1343	—	667	683	0.20	0.85	1.11	0.15	0.34
K8-1343–BSA	0.9	682	697	0.57	0.09	3.25	0.91	3.01
K8-1343–IgG	5.8	669	687	0.31	0.45	1.77	0.55	1.11
K8-1643	—	634	644	0.09	0.91	1.31	0.08	0.19
K8-1643–BSA	0.9	650	660	0.28	0.04	2.61	0.96	2.52
K8-1760	—	634	656	0.31	0.69	0.93	0.31	0.50
K8-1760–BSA	1.3	650	672	0.74	0.24	2.58	0.76	2.13
K8-1760–IgG	1.2	650	671	0.55	0.21	1.96	0.79	1.66
K1-204	—	381	480	32.5	1.00	—	—	32.5
K1-204–BSA	1.0	383	468	31.9	0.94	10.9	0.06	30.6
K7-545	—	425	545	26.2	1.00	—	—	26.2
K7-545–BSA	1.9	427	528	2.74	0.17	26.5	0.83	22.4

[a]All dashes in this column represent free dye (value is not applicable);
[b]All dashes in these two columns represent mono-exponential decay (value is not applicable).

in the pH range of 5.2–11.8. K8-1405 was the most important dye for pH-sensing applications because its pKa is in the physiological range and both the protonated and deprotonated forms are fluorescent. The reactive pH-sensitive labels can be easily coupled to antibodies and other proteins by using standard procedures. The conjugates are bright, and the pKa values of the IgG and BSA conjugates are similar to those for the free dyes. The spectral characteristics of representative dye–BSA and dye–IgG conjugates are shown in TABLE 4.

The pH-sensitive dyes (R^3 and/or R^4 are hydrogen; SCHEME 1) exhibited increased brightness in aqueous solutions compared with dyes of similar structure, where R^3 and/or R^4 are not hydrogen, and therefore they are better suited for labeling of low-molecular-weight biomolecules. Thus, for small-molecule labeling, K8-1665 is the best choice, with a QY of 33% which is even higher than that for Cy5 and Alexa 647.

Potential applications of the developed pH-sensitive labels are in biological, pharmaceutical, and biomedical research; clinical diagnostics; and high-throughput screening for the investigation of biological cells, membranes, and the role of intracellular pH in diverse physiological and pathological processes.

Probes for FLT Imaging and Homogeneous Fluorescence Polarization Assays of High-molecular-weight Analytes

Long-wavelength cyanine dyes such as Cy5 and Alexa 647 have FLTs on the order of 1 ns in aqueous solutions, and these FLTs do not change sufficiently in the presence of high-molecular-weight analytes such as proteins or when covalently bound to these analytes, which is a substantial limitation for use of these dyes for biomedical assays based on the FLT measurements. In general, the increase of the FLT for Cy5 and Alexa 647 is no more than 1.7 times. In contrast to these open-chain cyanines, the reported squaraine dyes have low FLTs in aqueous solution in the unbound state (<1 ns), but these FLTs significantly increase after binding to proteins. TABLE 5 reports the mean lifetimes (τ_{mean}) and the components (τ_1, f_1 and τ_1, f_2) for representative squaraine labels and protein conjugates in comparison to Cy5 and Alexa 647. For example, squaraine label

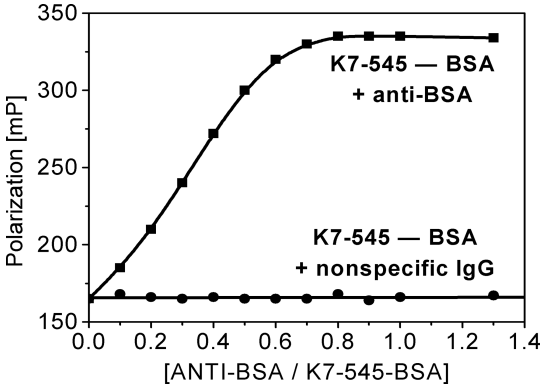


FIGURE 6. Changes in fluorescence polarization of K7-545–labeled BSA upon titration with anti-BSA and nonspecific IgG.

K8-1343 shows an increase of 10 times, from 0.3 ns in water to 3.0 ns when bound to BSA, and therefore this dye is a useful probe for FLT applications.

We have also developed SeTau markers such as K1-204 and K7-545, which are a series of naphthalene-based blue and green fluorescent probes and labels useful for the FLT- and polarization-based applications. Selected dyes of this series have FLTs up to 32 ns in aqueous solutions (TABLE 5). SeTau dyes are perfectly suited for use in homogeneous fluorescence polarization assays of high-molecular-weight antigens. A substantial polarization increase is observed upon binding of the fluorescently labeled antigen to the antibody. FIGURE 6 shows the changes in fluorescence polarization of K7-545–labeled BSA (molecular mass, ~66 kDa) upon titration with anti-BSA (molecular mass, ~150 kDa). The labeled BSA species still has a relatively low polarization of 165 mP and only upon addition of specific antibody the polarization increases gradually to its final value of 335 mP, which demonstrates the usefulness of this label for the measurement of high-molecular-weight antigens in a fluorescence polarization immunoassay.

Dark Quenchers

New reactive Super Quenchers of the SQ series, such as K8-1649, K8-1902, and K8-2602, absorb in the 500- to 800-nm spectral range with high extinction coefficients (TABLE 1). They exhibit no residual fluorescence and are perfectly suited for covalent labeling of proteins, peptides, and oligonucleotides for use in FRET and real-time polymerase chain reaction–based applications. These dyes are available as amine-reactive NHS esters and thiol-reactive maleimides. Thus, the reactive label K8-2602 has broad absorption band between 600 and 800 nm (FIG. 1), with maximum at 756 nm in water and an extinction coefficient as high as 200,000 $M^{-1} \cdot cm^{-1}$.

Conclusions

A series of bright and photostable probes and labels, FLT markers, and dark quenchers has been developed. They are suitable for biomedical assays based on fluorescence intensity, polarization, lifetime, or FRET.

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Conflict of Interest

The authors declare no conflicts of interest.

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