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Improved Photostability and Fluorescence Properties through Polyfluorination of a Cyanine Dye

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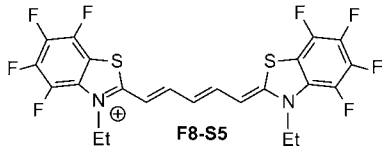
ABSTRACT



A polyfluorinated cyanine dye has been synthesized and characterized. Compared with the nonfluorinated analogue, the dye exhibits significantly reduced aggregation in aqueous media, enhanced fluorescence quantum yield, greater resistance to photobleaching upon direct irradiation, and reduced reactivity toward singlet oxygen. All of these properties are favorable for use of cyanine dyes as fluorescent labels and point toward fluorination as a general strategy for improving performance in imaging applications.

Cyanine dyes have found widespread use as fluorescent labels for biomolecules. The appeal of this class of fluorophores derives from their straightforward syntheses, broad wavelength tunability, large molar extinction coefficients, and moderate-to-high fluorescence quantum yields.[1] Cyanine dyes are currently available with absorption and fluorescence spectra that span the visible region. However, two common problems encountered by cyanine dyes and numerous other fluorophores are (i) their susceptibility to form nonfluorescent aggregates[2] and (ii) their tendency to undergo photobleaching.[3] This report describes the synthesis and preliminary characterization of a polyfluorinated cyanine dye with substantially improved resistance to both aggregation and photobleaching.

Synthesis of the octafluorinated thiadicarbocyanine dye **F8-S5** was accomplished in two steps from the precursor[4] 2-methyl-4,5,6,7-tetrafluorobenzothiazole. Alkylation of the heterocycles was achieved by reaction with diethyl sulfate and the resulting benzothiazolium salt was converted directly to the cyanine dye without isolation (Scheme 1).

We compared the photophysical and photochemical properties of **F8-S5** with those of its nonfluorinated analogue, **S5**. Fluorination suppresses the extinction coefficient (ϵ) for the dye in methanol by 50%: $\epsilon_{651} = 260{,}000\ M^{-1}\ cm^{-1}$ for **S5** versus $\epsilon_{651} = 130\,000\ M^{-1}\ cm^{-1}$ for **F8-S5**. No shift in the absorption wavelength maximum was observed. A polyfluorinated cyanine dye based on benzimidazole heterocycles exhibited a slight (6 nm) hypsochromic shift of its absorption spectrum.[5] Similarly, fluorination of distyrylbenzene led to only modest shifts in absorbance spectra relative to the parent hydrocarbon.[6]

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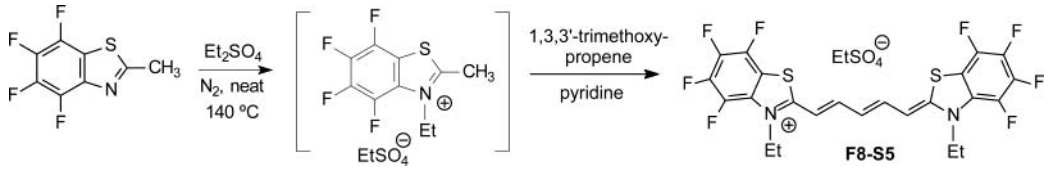
Scheme 1. Synthesis of Polyfluorinated Cyanine Dye **F8-S5**



While fluorination suppresses the extinction coefficient for the dye, the fluorescence quantum yield (Φ_f) in methanol is approximately 13% higher for **F8-S5**: $\Phi_f = 0.17$ for **F8-S5** versus 0.15 for **S5**.

Significant differences between the two cyanine dyes emerge when comparing their properties in aqueous solutions. Figure 1 illustrates absorption spectra for the two dyes

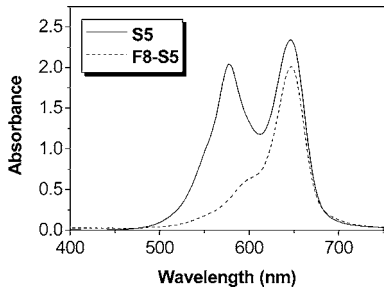


Figure 1. UV−vis spectra recorded for 25 μM **S5** and **F8-S5** cyanine dyes in pure water.

recorded in pure water. The shape of the spectrum for **F8-S5** is nearly identical with that observed in methanol, whereas the spectrum for **S5** has an additional peak at shorter wavelength. This peak is characteristic of assembly of cyanine dyes into noncovalent dimers and higher aggregates.[2] **S5** dimerizes in pure water with an equilibrium constant $K = 6.7 \times 10^4\ M^{-1}$.[2b] On the basis of concentration dependence studies, we estimate that the equilibrium constant for dimerization of **F8-S5** is at least 10-fold lower (data not shown).

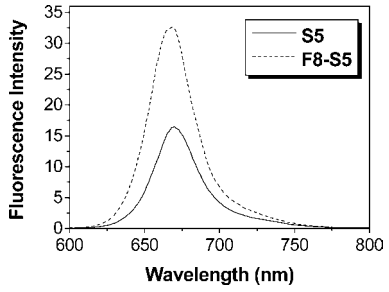


Figure 2. Fluorescence spectra recorded for 5.0 μM **S5** and **F8-S5** in aqueous buffer (10 mM sodium phosphate, pH 7, 20% methanol included to suppress adsorption to cuvette). Samples were excited at 545 nm.

The impact of dye aggregation is evident in the fluorescence spectra shown in Figure 2: the intensity is nearly twice as high for the nonaggregating fluorinated dye as for **S5**. Aggregated cyanines in which the absorption is shifted to shorter wavelengths ("H-aggregates") exhibit quenched fluorescence, diminishing their utility as probes and labels; fluorination of the dye suppresses the tendency to aggregate and leads to higher fluorescence intensities.

Further evidence of **F8-S5**'s reduced tendency to aggregate comes from experiments with DNA. We previously reported that **S5** assembles into helical aggregates within the minor groove of duplex DNA nanotemplates.[7] These aggregates are formed from **S5** dimers aligned end-to-end within the groove of the DNA and the orbital mixing within the dimer results in a 60 nm blue shift in the dye absorption spectrum, yielding a new peak at 590 nm (Figure 3, left). In this

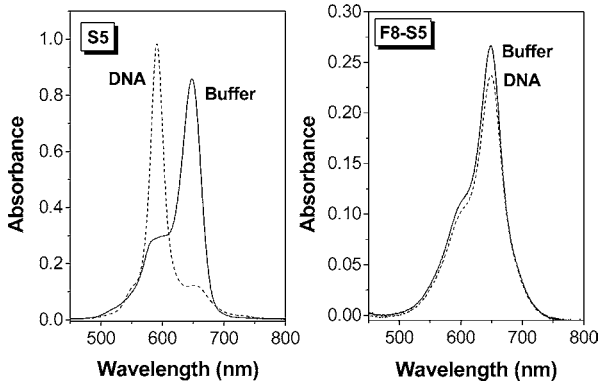


Figure 3. UV−vis spectra recorded for **S5** (left) and **F8-S5** (right) in the presence or absence of duplex DNA in aqueous sodium phosphate buffer (10 mM, pH 7.0, 20% methanol). [Dye] = 5.0 μM; [DNA] = 20 μM base pairs. [Poly(dI-dC)]$_2$ was used as the DNA template. Solid line = buffer; dashed line = DNA.

experiment, the DNA *promotes* assembly of the dimer, since the dimer band at 590 nm is significantly suppressed in the absence of DNA. In contrast, the fluorinated dye exhibits only a slight decrease in its absorption intensity in the presence of DNA and no enhanced absorbance in the region of the dimer band (Figure 3, right).

Cyanine dye photobleaching can arise from multiple pathways, including (1) reaction with singlet oxygen, generated in situ, and (2) reaction with hydroxyl radical produced

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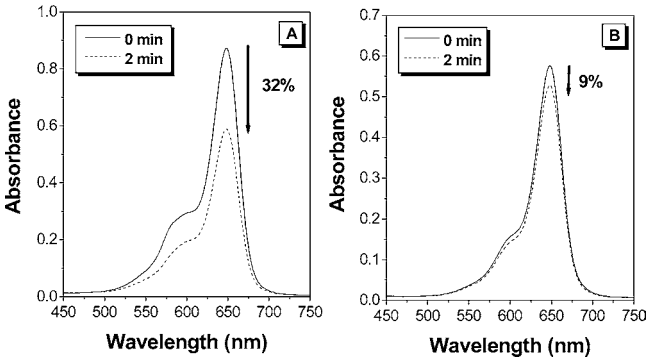

Figure 4. UV−vis spectra recorded before and after 2 min of irradiation with visible light for 5.0 μM **S5** (A) and **F8-S5** (B) in aqueous sodium phosphate buffer (10 mM, pH 7.0) and 20% methanol. The percent of photobleaching was calculated from the loss in absorbance at 648 nm.

through Fenton-like chemistry.[8,9] Figure 4 compares the photobleaching of **F8-S5** and **S5**, as monitored by UV−vis spectroscopy. The samples were irradiated with the filtered (λ > 550 nm) output of a 150 W Hg(Xe) lamp for 2 min. As shown in Figure 4, the nonfluorinated dye loses 32% of its absorption intensity during the irradiation, while the fluorinated dye only loses 9%. Similar results were obtained when the fluorescence intensity of the dye solutions was monitored: a 24% decrease was observed for **S5** while the intensity only decreased by 6% for **F8-S5** (Figure S1). Even after accounting for the lower extinction coefficient, fluorination significantly stabilizes the cyanine dye against photobleaching.

Singlet oxygen can be produced in situ by irradiation of dyes as shown in Scheme 2. The intersystem crossing yields

Scheme 2. Photochemical Production of Singlet Oxygen ($^1O_2$) from Fluorescent Dyes

$$^1\text{Dye} \xrightarrow{h\nu} {}^1\text{Dye}^* \xrightarrow[\text{Intersystem Crossing}]{} {}^3\text{Dye}^* \xrightarrow{^3O_2} {}^1\text{Dye} + {}^1O_2^*$$

for cyanine dyes are usually quite low, meaning the quantum yields for singlet oxygen production will be correspondingly low; nevertheless, cyanines are known to react rapidly with singlet oxygen.[10]

Singlet oxygen can add to conjugated dienes[11] and an analogous reaction with cyanine dyes would result in loss of conjugation, i.e. photobleaching. However, Kanofsky and Sima reported that quenching of singlet oxygen by cyanine dyes decreased as the oxidation potential of the dye decreased.[10] While we have not measured the oxidation potential of **F8-S5**, it is likely that the potential is considerably more positive than that of **S5** due to the eight electron-withdrawing fluorine substituents. This suggests that the fluorinated dye should be less susceptible to reaction with singlet oxygen, a prediction we tested using the photosensitizer eosin Y. This dye has a high quantum yield for production of singlet oxygen (0.57 in water)[12] and allows singlet oxygen to be introduced to the system without excitation of the cyanine dyes.

Light from the Hg(Xe) arc lamp was filtered to permit selective irradiation of eosin Y in the presence of the cyanine dyes, and the effects on the dye absorbance and fluorescence spectra were measured. Figure 5 shows that irradiation of

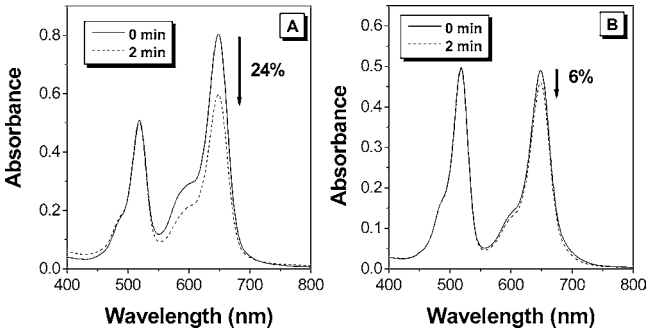

Figure 5. Eosin Y-sensitized photobleaching of **S5** (A) and **F8-S5** (B). Samples were irradiated with visible light (λ < 550 nm, UV cutoff) for 2 min. [Dye] = [eosin Y] = 5.0 μM.

eosin Y results in 24% bleaching of **S5** but only 6% bleaching of **F8-S5**. (Only 8% and 3% bleaching were observed for **S5** and **F8-S5**, respectively, under these conditions in the absence of eosin Y.) Similar results are found in the fluorescence spectra: 21% loss for **S5** but only 4% loss for **F8-S5** (Figure S2). These results demonstrate that the fluorinated dye is significantly less susceptible to reaction with singlet oxygen that might be produced in situ during irradiation.

Finally, we considered the possibility that the difference in photostability for the two dyes was due to the presence of different counterions, iodide for **S5** versus ethyl sulfate for **F8-S5**. Thus we synthesized **S5** with an ethyl sulfate counterion but its photostability, UV−vis spectrum, and fluorescence quantum yield were virtually identical with those of the iodide salt (data not shown). Therefore the improved photostability of the fluorinated dye can be attributed to fluorination of the benzothiazole heterocycles.

There is one other report in the literature of a polyfluorinated cyanine dye.[5] That dye was based on benzimidazole heterocycles rather than benzothiazole. The effect of fluorination on photostability and fluorescence properties were not reported.

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A number of reports concerning stabilizing fluorescent dyes through encapsulation within various receptors such as cyclodextrins[13] have appeared. Our results suggest that polyfluorination of cyanine dye heterocycles provides an alternative or complementary strategy for improving their performance in fluorescence imaging applications due to reduced aggregation and photobleaching susceptibility. We are currently preparing additional fluorinated cyanine dyes to assess the generality of this approach and for attachment to biomolecules for imaging studies.

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Supporting Information Available: Detailed descriptions of experimental procedures, synthetic details and spectral data for the ethyl sulfate salts of **F8-S5** and **S5**, and fluorescence photobleaching data. This material is available free of charge via the Internet at http://pubs.acs.org.

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