CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200801104

Water-Soluble Nile Blue Derivatives: Syntheses and Photophysical Properties

Jiney Jose, Yuichiro Ueno, and Kevin Burgess*[a]

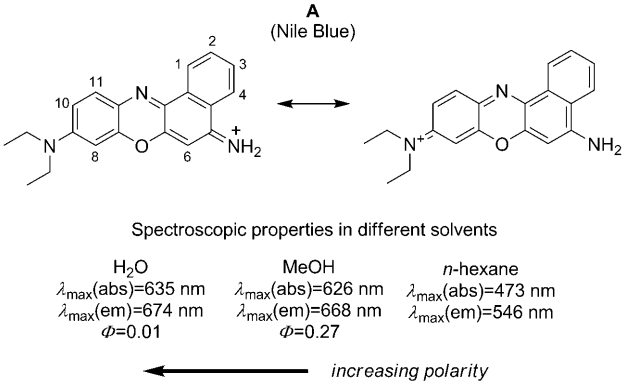
Abstract: Four water-soluble 2-hydroxy-Nile Blue derivatives, **1a**, **1b**, **2a**, and **2b**, were prepared by condensation reactions performed under relatively mild conditions (90 °C, *N*,*N*-dimethylformamide with no added acid). These fluorescent probes had more favorable fluorescence characteristics than two known water-soluble Nile Blue derivatives. Specifically, they were superior to the known dyes with respect to their quantum yields in aqueous media and the sharpness of their fluorescence emissions. Concentration-dependant UV absorption and fluorescence emission studies indicated that the dyes did not aggregate in aqueous solution at concentrations of less than 1–4 μM. The new water-soluble materials **1a**, **1b**, **2a**, and **2b** emit in a desirable region of the fluorescence spectrum ($\lambda = 670$–675 nm). Overall they are potentially interesting for labeling biomolecules in aqueous environments.

Keywords: biochemical markers · dyes/pigments · fluorescence · Nile Blue · water-soluble groups

Introduction

Nile Blue, **A**, is a fluorescent probe that has been known for over 110 years.[1,2] In polar media its absorption and emission maxima shift to the red, which is indicative of stabilized charge separation in the excited state; consequently, this dye has been used to monitor events that depend upon solvent polarity.[3–5] It has also been used for fluorescence resonance energy transfer (FRET) studies.[6,7] Nile Blue tends to have a higher affinity for cancerous cells than healthy ones[8] and it is a photosensitizer for oxygen;[9,10] these two properties together can be useful in photodynamic therapy.[11] However, two properties of Nile Blue in aqueous media are limiting for many applications, specifically 1) low solubility and 2) low quantum yield.

Some work has been published on Nile Blue derivatives with improved water solubilities.[12–15] The aggregation of inherently flat, lipophilic aromatic dyes is disfavored when they are functionalized with water-solubilizing substituents and their quantum yields can improve as a result. Consequently, Nile Blue derivatives with hydrophilic groups can have improved solubilities *and* fluorescence outputs. Derivatives **B** and **C** are the most interesting probes to arise from these studies.[12] Their quantum efficiencies are improved by as much as a factor of ten, however, they have no carboxylic acid handle for attachment to biomolecules, and the sharpness of their emissions broadens as the solvent is changed from methanol to water, which is perhaps indicative of aggregation. Other work involved the incorporation of groups that offer only incrementally enhanced water solubilities,[13] lack of quantum yield data,[14] and/or no experimental procedures for the syntheses.[14,15]



This paper reports the syntheses of 2-hydroxy Nile Blue derivatives **1** and **2** and compares their fluorescence properties with those of **B** and **C**. For both probes, a 2-hydroxy substituent was incorporated to enhance water solubility and other hydrophilic groups are situated on both ends of the molecule to reduce the potential for aggregation. Not all

[a] J. Jose, Y. Ueno, K. Burgess
Department of Chemistry
Texas A&M University, Box 30012
College Station, TX, 77841 (USA)
Fax: (+1) 979 845-8839
E-mail: burgess@tamu.edu

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200801104.

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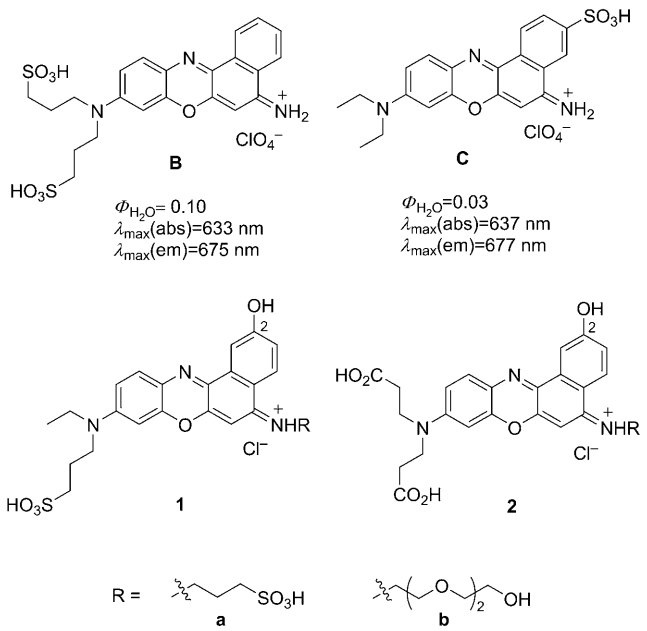


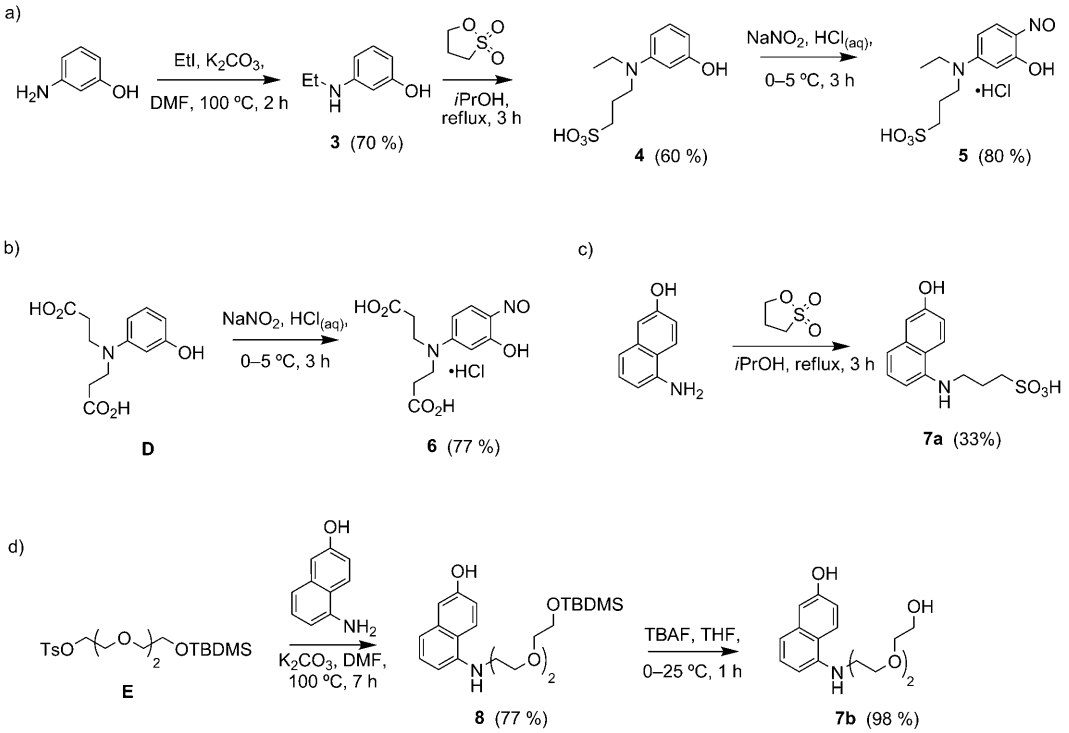
possible applications of these dyes require functional groups for attachment to biomolecules but many do, and compounds **2a** and **2b** were designed for that purpose.

Results and Discussion

Syntheses of functionalized aminophenol and aminonaphthol components: The western part of target compounds **1** and **2** were formed from functionalized nitrosophenols; these were prepared as outlined in Scheme 1. Nitroso compound **6** was formed by nitrosylation of phenol **D**, a starting material previously used in our laboratories for syntheses of Nile Red derivatives.[16] 3-(*N*-ethylamino)phenol **3** and derivative **4** have been previously described in a patent that gives the experimental procedures.[17] Nitrosylation of **4** gave nitroso compound **5**. Both the aminonaphthol components **7** required for the eastern half of these molecules were made by alkylation reactions. Compound **7a** was obtained by alkylation with propane sultone (Scheme 1c). The triethylene glycol derivative **E** was conveniently made in a few steps from the parent diol, then this was used to N-alkylate 5-amino-2-naphthol as shown (Scheme 1d).[18–20]

Syntheses of water-soluble Nile Blue derivatives 1 and 2: Previous syntheses of Nile Blue derivatives required relatively high temperatures and/or strong acids. Compounds **1** and **2** were synthesized by condensation at a relatively low temperature (90 °C) without any additional acids. The blue products were isolated by using medium-pressure liquid chromatography (MPLC) on a reverse-phase C18 column (Scheme 2).

Spectroscopic properties of the Nile Blue derivatives: The electronic spectra (Figure 1) of the dyes were recorded in methanol (as a representative polar organic solvent), in 0.1 M phosphate buffer at pH 7.4 (see Table 1), in the same buffer but with 3% Triton X-100, and in 0.1 M borate buffer at pH 9. Only **1a** and **2a** were soluble in MeOH; data for **1b** and **2b** could not be obtained in this medium. The effects



Scheme 1. DMF = *N*,*N*-dimethylformamide, TBDMS = *tert*-butyldimethylsilyl, TBAF = tetra-*n*-butylammonium fluoride.

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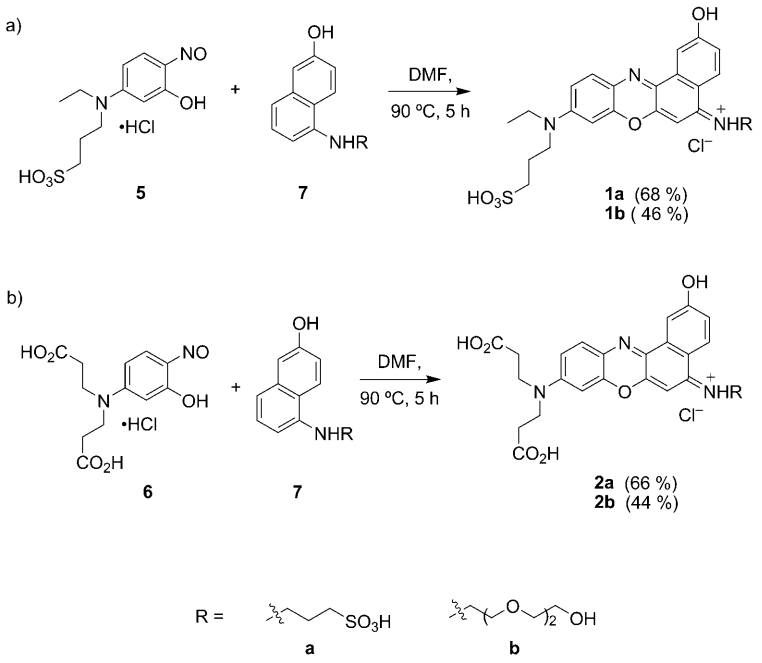

Scheme 2.

of adding 3% Triton X-100 to the medium are ambiguous; this reagent changes the solvent polarity, but might also prevent aggregation effects.[4,21,22] In borate buffer at pH 9.0, the phenolic hydroxyl of the dyes are predominately in the anionic form.

All the dyes had absorption maxima between $\lambda = 628$ and 632 nm under all the conditions described above; consequently, there is little variation in this parameter with solvent polarity. Extinction coefficients for the molecules, however, were in the range 14400–64100. For **1a**, **2a**, and **1b**, the maximum values correspond to the media that included 3% Triton X-100; such enhancement effects have been observed for fluorescent dyes,[21,22] including Nile Blue.[4] Fluorescent emission maxima for the dyes varied between $\lambda = 662$–677 nm. The fact that compounds **1a** and **2a** had fluorescence emission maxima in MeOH that were within 9 nm of the values obtained in aqueous buffers means that the solvatochromic effects for these two materials are much less than for Nile Blue. Furthermore, the lack of significant

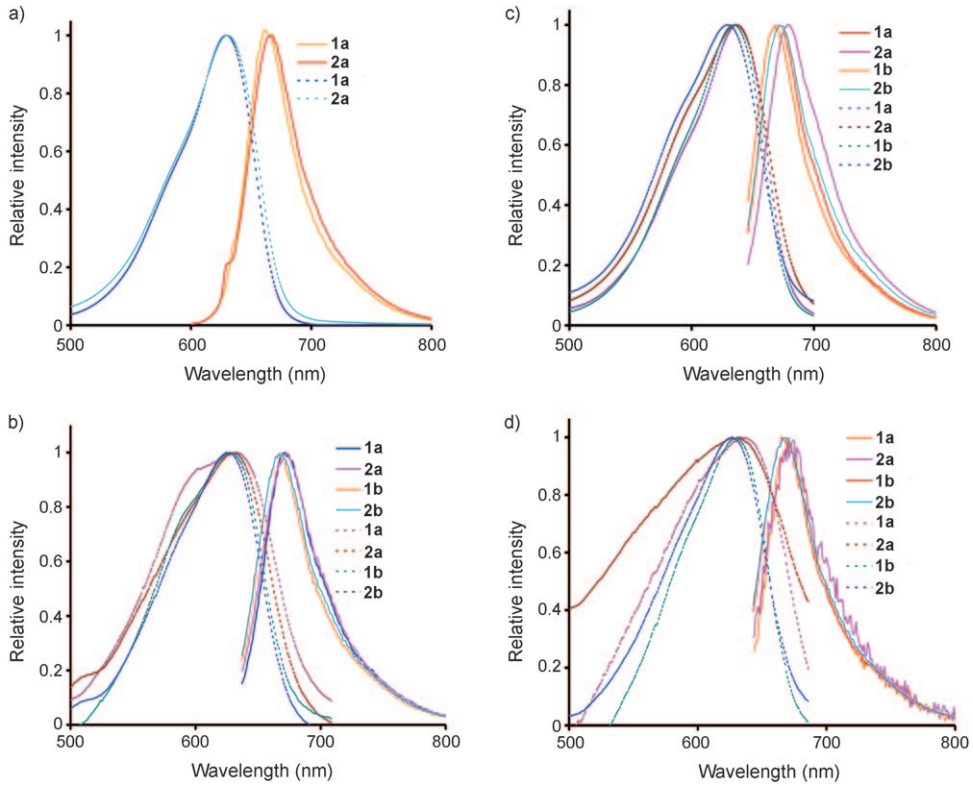

Figure 1. Absorption (dashed lines) and fluorescence (solid lines) of a) **1a** and **2a** in methanol and **1a,b** and **2a,b** in b) phosphate buffer (pH 7.4), c) phosphate buffer (pH 7.4) with 3% Triton X-100, and d) borate buffer (pH 9.0). All dyes (2×10^{-6} M) were excited at their corresponding λ_{max}.

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Table 1. Spectroscopic properties of Nile Blue and its derivatives under different conditions.[a]

Dye	λ_{abs} [nm]	ε [$M^{-1}cm^{-1}$]	λ_{em} [nm]	fwhm [nm]	Φ[b]	Solvent
1a	628	14400	662	47	0.56	MeOH
1a	630	42400	671	52	0.14	PB
1a	631	51200	669	49	0.24	TX
1a	630	28400	670	56	0.02	BB
2a	629	58800	666	50	0.32	MeOH
2a	630	30300	670	58	0.10	PB
2a	629	64100	669	56	0.11	TX
2a	631	34500	670	73	0.02	BB
1b	629	33600	671	55	0.14	PB
1b	628	44400	670	47	0.23	TX
1b	630	21800	672	51	0.13	BB
2b	632	38100	673	56	0.13	PB
2b	631	14700	672	54	0.26	TX
2b	630	38000	670	54	0.08	BB
A[c]	635	4000	674	115	0.01	water
B[c]	633	36000	675	86	0.10	water
C[c]	637	11000	677	93	0.03	water

[a] PB: Phosphate buffer (pH 7.4), TX: 3% Triton X-100 in phosphate buffer (pH 7.4), BB: borate buffer (pH 9.0). [b] Standard used for quantum yield measurement: Nile Blue in MeOH ($\Phi = 0.27$), quantum yield and extinction coefficient experiments (at 10^{-6} M) were repeated three times. [c] Values obtained from ref. [12].

variations between the emission wavelengths in various buffers indicates that changing the pH away from physiological levels and adding lipophilic cosolvents have little effect on these dyes. The sharpness of the fluorescent emissions are expressed in terms of full width at half maximum peak heights (fwhm; in which smaller is sharper). Dyes **1** and **2** emitted with sharper fluorescence peaks than Nile Blue or the more water-soluble forms **B** and **C** (data shown in Table 1 for these dyes is taken from the literature). Furthermore, in aqueous media the quantum yields for these emissions for **1** and **2** were in all cases better than for Nile Blue and its derivatives **B** and **C**.[12]

Figure 2 outlines experiments performed to explore the aggregation of the dyes in aqueous media. Plots of the normalized UV absorbance versus concentration reveal that the λ_{max}(abs) for compound **1a** at 4 μM occurs at 671 nm, with an inflection on the blue side of the peak at approximately 600 nm (Figure 2a). This inflection point grew as the concentration of the dye was increased; at 16 μM there are two distinct absorption maxima, and at higher concentrations, the shorter wavelength absorption becomes dominant. Figure 2b shows that at concentrations of up to 4.0 μM the absorbance of **1a** varies in a near-linear way with concentration. Above that concentration, the absorbance deviates markedly from linear concentration dependence. Overall, these data may be interpreted to mean that the dye is aggregating at concentrations above around 4.0 μM. Similar analyses using UV absorption indicate that **1a** deviates from Beer–Lambert behavior above this concentration. Probably the dyes are forming fluorescent J-aggregates at concentrations above about 4.0 μM, rather than the nonfluorescent H-forms. Analyses for dyes **1b**, **2a**, and **2b** (Figure 2c–h) indicate very similar behavior. Concentration versus absorbance studies indicate that these materials tend to aggregate above 4.0 μM.

Finally, Nile Blue derivative **2a** was used to label ovalbumin by activation of the dicarboxylic acids on the dye by using *N*-hydroxysuccinimide and *N*,*N*′-diisopropylcarbodiimide in DMF, followed by addition of this activated probe to the protein in 0.1 M aqueous $NaHCO_3$ (pH 8.3). The dye/protein ratio was calculated[25] to be 1.1 if five equivalents of dye was used; this corresponds to a 22% labeling efficiency. This sample was used to obtain the spectral data shown in Figure 3a. The wavelengths for the absorption and fluorescence maxima for free **2a** and the **2a**–ovalbumin conjugate were observed to be almost identical, but the fluorescence intensity was much less.

One application of Nile Blue derivatives is to measure protein concentrations; this is possible because the fluorescence intensities of Nile Blue derivatives tend to increase with protein concentration.[3] However, one limitation of this method is that the solubility of Nile Blue derivatives can be problematic. Herein, Nile Blue derivative **2a** was mixed with increasing concentrations of ovalbumin in phosphate buffer at pH 6.8. The fluorescence data for this set of experiments are shown in Figure 3b. The measurements were performed at different pH values because in one case a covalent interaction was formed by using a protocol at pH 8.3, whereas the other was a simple addition at a more standard pH. The fluorescence intensity of **2a** increased when the protein was added. These increases were small, but the concentrations of ovalbumin were only varied between 3 and 12 μM, that is, small changes in protein concentration that are hard to detect. Furthermore, unlike in some previous works with lipophilic Nile Blue derivatives, use of the water-soluble form **2a** circumvented the need for any detergent additives.

Conclusion

The Nile Blue derivatives reported here have sharper fluorescence emissions (fwhm = 30 nm smaller), and improved quantum yields in phosphate buffer at pH 7.4 relative to the known water-soluble Nile Blue derivatives **B** and **C**. They are formed by condensation reactions that do not require additional acids or very harsh reaction conditions (DMF, 90 °C); this is in marked contrast to the syntheses of most other Nile Blue derivatives. Preparative HPLC purification of the products was not necessary; they were isolated by using reverse-phase MPLC with acetonitrile/water as the eluent. The phenolic OH functionalities of **1** and **2** almost certainly increase the water solubilities of these compounds. Alternatively, the phenolic group provides a potential avenue for further derivatization of the dyes (e.g., through triflation and organometallic couplings, or for attachment of a handle to enable **1** to be conjugated to proteins). Three other groups that promote water solubility were included in these studies: a sulfonic acid, dicarboxylic acids, and a triethylene glycol fragment. Despite this, the fluorescence properties of the dyes, and presumably their aggregation states at elevated concentrations, did not vary significantly.

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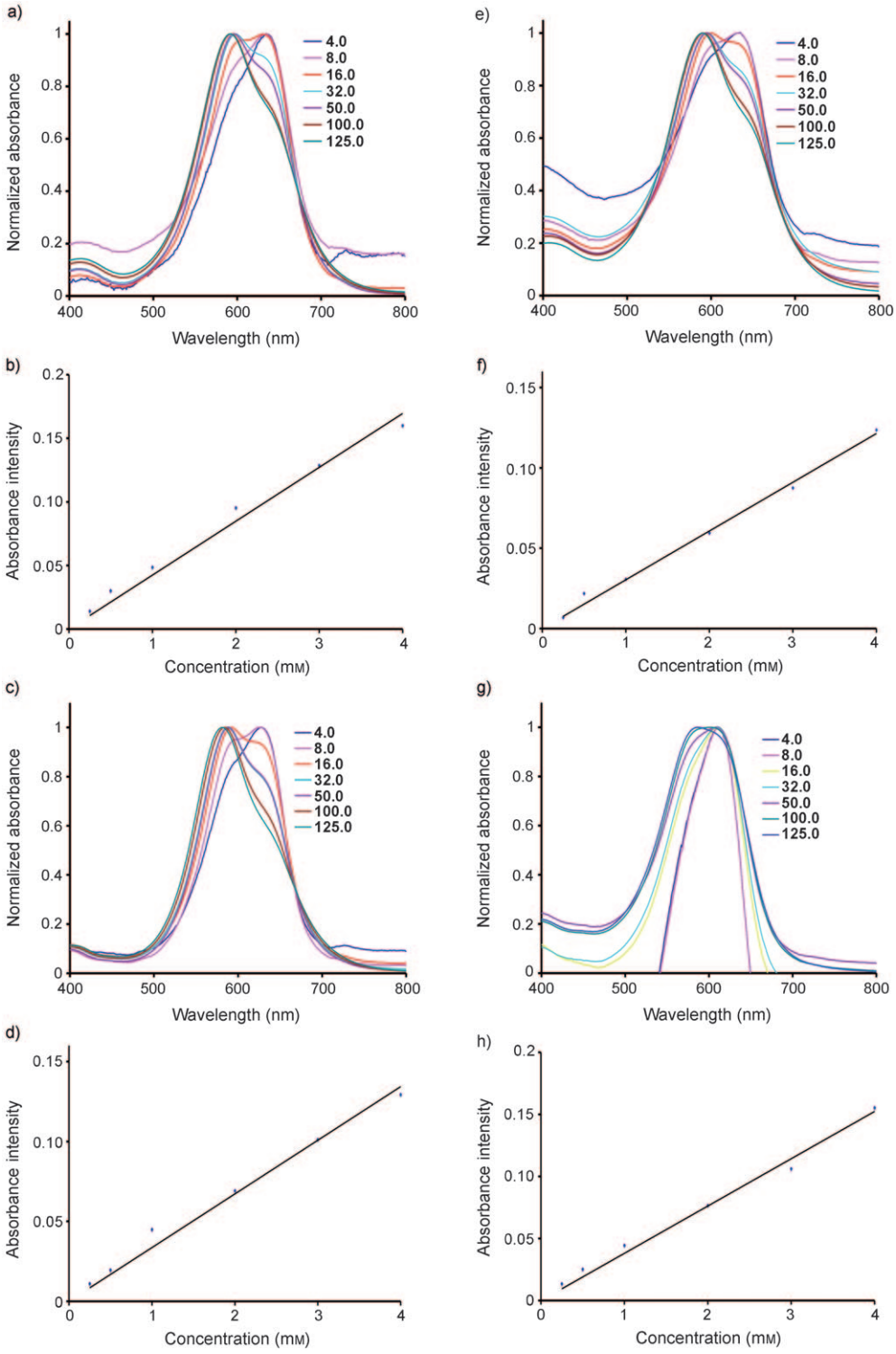

Figure 2. Aggregation studies. Normalized absorption for various concentrations of **1a** (a), **1b** (c), **2a** (e), and **2b** (g) in phosphate buffer at pH 7.4, and plots of absorbance intensity vs. concentration of **1a** (b), **1b** (d), **2a** (f), and **2b** (h).

All the dyes showed little tendency to aggregate below 1–4 μM; this characteristic would tend to make them useful for biochemical studies when used in relatively dilute solutions, but would exclude applications in which quantization is required at higher concentrations. Probably the most useful spectroscopic parameter of the dyes is their fluorescence at relatively long wavelengths, $\lambda = 670$ to 680 nm, in aqueous media. Probes that emit above $\lambda = 650$ nm are relatively few, yet they tend to be the most useful ones for tissue and intracellular imaging applications.[23,24]

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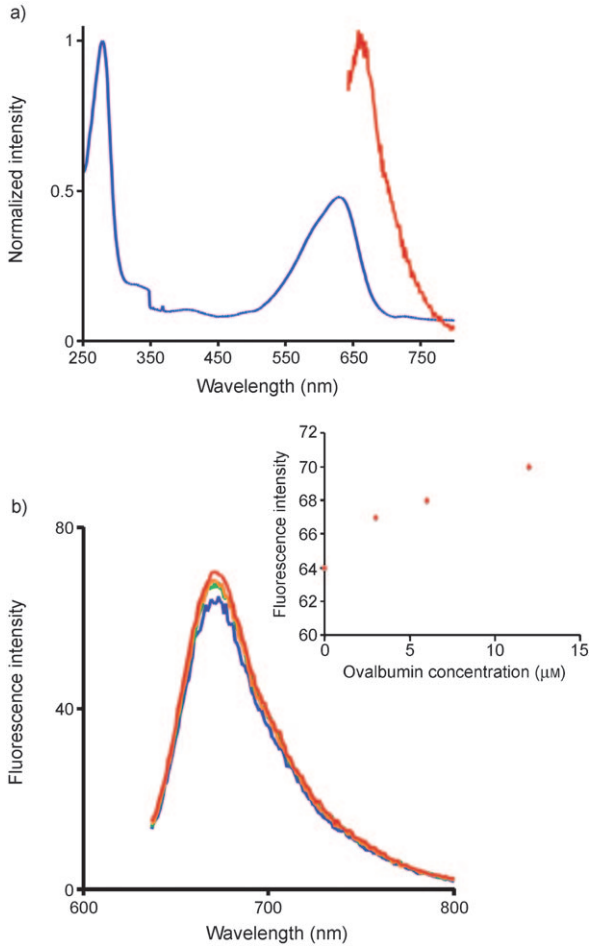

Figure 3. a) Absorbance (blue) and fluorescence (red) spectra of **2a**–ovalbumin in 0.1 M phosphate buffer (pH 7.4). b) Fluorescence spectra of **2a** (5×10^{-7} M) and blue: 0, green: 3.0, orange: 6.0, and red: 12.0 μM ovalbumin in phosphate buffer (pH 6.8), $\lambda_{ex}=630$ nm. Inset: Variation in the fluorescence intensity of **2a** vs. ovalbumin concentration.

Experimental Section

General procedure for the synthesis of Nile Blue derivatives 1a,b and 2a,b: Nitroso compound **5** or **6** (0.3 mmol) was dissolved in dry distilled DMF (5 mL) and 5-amino-2-naphthol **7a** or **7b** (0.3 mmol) was added with stirring. The reaction mixture was heated to 90 °C for 5 h and then cooled to RT. The DMF was removed under reduced pressure and the residual material was dissolved in water (10 mL). This solution was filtered to remove solid impurities and the filtrate was purified by using reverse-phase MPLC and eluting with 1:1 CH_3CN/H_2O to afford the corresponding Nile Blue derivative as a dark blue solid.

Procedure for conjugation of ovalbumin protein to 2a: Nile Blue derivative **2a** (8.0 mg, 0.014 mmol) was activated by using *N*-hydroxysuccinimide (10.0 mg, 0.084 mmol) and *N*,*N*′-diisopropylcarbodiimide (14.0 mg, 0.1 mmol) in DMF (0.4 mL) at 25 °C for 24 h. The activated dye solution (17 μL, 0.6 μmol) was added to ovalbumin (5.0 mg, 0.12 μmol) in aqueous $NaHCO_3$ buffer (0.1 M, pH 8.3, 1.0 mL) and stirred at 25 °C for 30 min. Purification of the protein–dye conjugate was performed by using a Sephadex G25M column.

Measurement of the fluorescence intensity variation of 2a with increasing concentrations of ovalbumin: A stock solution of ovalbumin (1.0 mg mL^{-1}) in phosphate buffer at pH 6.8 was prepared in a 25 mL volumetric flask. Dye **2a** (stock solution, 1.0 mL, 2.0×10^{-6} M, pH 6.8), phosphate buffer (pH 6.8), and an appropriate amount of ovalbumin stock solution was added to a cuvette to obtain a final volume of 4.0 mL. The fluorescence intensities of the sample and the blank (with no ovalbumin stock solution) were measured at $\lambda=671$ nm at 25 °C. All solutions were excited at $\lambda=630$ nm.

Acknowledgements

We thank Dr. Michael J. Collins and CEM Corporation for their support with microwave technologies (which was used in exploratory studies), and TAMU/LBMS-Applications Laboratory directed by Dr. Shane Tichy for assistance with mass spectrometry. Support for this work was provided by The National Institutes of Health (GM72041) and by The Robert A. Welch Foundation.

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Received: June 7, 2008
Revised: September 29, 2008
Published online: November 21, 2008

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