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Synthesis and Characterization of a Solvatochromic, Lipophilic Fluorescent Oxazone: 1-Pentyl-7-dimethylamino-3H-phenoxazine-3-one[1]

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Olivetol and 4-nitroso-*N,N*-dimethylaniline were combined under reflux in acetic acid to produce 1-pentyl-7-dimethylamino-3H-phenoxazine-3-one (1-PDMPO). The fluorescent behavior of the purified compound in a variety of solutions and samples was investigated. Excitation and emission spectra in pure solvents and mixtures of solvents demonstrate solvatochromism, indicating that the fluorescence response of this compound is affected by its environment. Nonpolar, aprotic solvents as well as protic, polar solvents diminish fluorescent emission in the spectral regions examined, and trends in the fluorescence decay lifetimes measured in five pure solvents are consistent with these intensity changes. The anisotropy excitation spectrum taken in glycerol for 1-PDMPO at −15°C appears to be consistent with the presence of a single electronic state upon excitation, with anisotropy values approaching 0.35 over 400–600 nm. Fluorescence emission is also diminished at low acid concentrations in methanol, with smaller decreases observed in more highly concentrated basic solutions. Emission peaks in aqueous sodium dodecyl sulfate solutions, extruded egg phosphatidylcholine vesicles, and fatty acid free bovine serum albumin suspensions all lie above 600 nm, with emission in the albumin suspension displaying a broad shoulder extending to 800 nm. The fluorescent properties of this compound suggest that it or structural homologues may have utility as fluorescent biological probes.

KEY WORDS: 1-Pentyl-7-dimethylamino-3H-phenoxazine-3-one; fluorescent oxazone; solvatochromism.

INTRODUCTION

Fluorimetry at wavelengths greater than 500 nm might reasonably be expected to offer several advantages: decreased scattering of light due to the inverse fourth-power intensity–wavelength relationship, a reduction in background due to the lack of emission from biological chromophores, and the possibility of developing cheap and simple instrumentation [1,2]. However, complete exploitation of these desirable characteristics requires the development and characterization of fluorescent probes and dyes with useful excitation and emission responses in this spectral region [3]. With these considerations in mind, we set out to synthesize and characterize fluorescent compounds which might show desirable emission and excitation characteristics, as well as lipophilic and solvent sensitive properties likely to enhance their use in characterizing biological and biomimetic structures.

Compounds showing a high sensitivity to the environment or solvent in their absorption and emission spectra are termed *solvatochromic*. Common examples used in biological fluorimetry include the probes PRO-

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DAN, LAURODAN, and Nile Red (Fig. 1). For all of these neutral probes, resonance structures can be drawn which suggest that charge transfer can take place between an electron pair-donating amine nitrogen and an electron-accepting carbonyl oxygen, and such charge transfer would be expected to enhance solvatochromic sensitivity in the excited state. Earlier work based on this model has been presented previously for Nile Red [4,5]. By analogy to this compound, it seemed appropriate to investigate the synthesis of amine-substituted oxazones possessing acyl chains likely to enhance solubility in membranes and lipophilic proteins. Review of the chemical literature showed that 7-dimethylamino-3H-phenoxazine-3-one derivatives (Fig. 2) had previously been synthesized from nitrosoanilines and resorcinol derivatives [6]. Given this procedure and the commercial availability of olivetol, a resorcinol derivative possessing a pentyl acyl substituent, 1-pentyl-7-dimethylamino-3H-phenoxazine-3-one (1-PDMPO) was prepared to examine its fluorescent behavior in a variety of solvents and samples. Below, experimental details relating to the synthesis and purification of 1-PDMPO are reported, along with characterization of its fluorescence behavior in several systems.

EXPERIMENTAL

Solvents and Reagents. Solvents and reagents used in the synthesis and purification of 1-PDMPO were all of reagent grade or better and used without further purification. In the initial synthetic attempts, 4-nitroso-*N,N*-dimethylaniline and olivetol (Lancaster) were used, while in later preparations purified olivetol prepared in the laboratory of Dr. Harold Pinnick of Bucknell was substituted without noticeable effect. Organic solvents used in fluorimetry were all HPLC or "Optima" grade (Burdick and Jackson, Fisher) and used without further purification. No fluorescent emission or excitation was observed in these pure solvents over the wavelength regions reported. Sodium dodecyl sulfate (Sigma), egg phosphatidylcholine (Avanti), and fatty acid free bovine serum albumin (Sigma) were all used as received. Doubly deionized water was used in the preparation of all aqueous and Tris (Sigma) buffer solutions. Egg phosphatidylcholine vesicles were prepared using a Lipex (Vancouver, BC) extruder according to the method of Hope *et al.* [7].

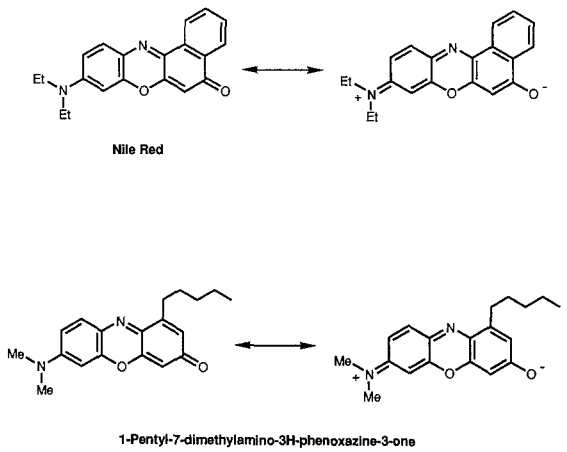

Fig. 1. Resonance structures demonstrating charge transfer in oxazone derivative and Nile Red. Conjugation of electron-donating and electron-accepting substituents enhances solvatochromic effects.

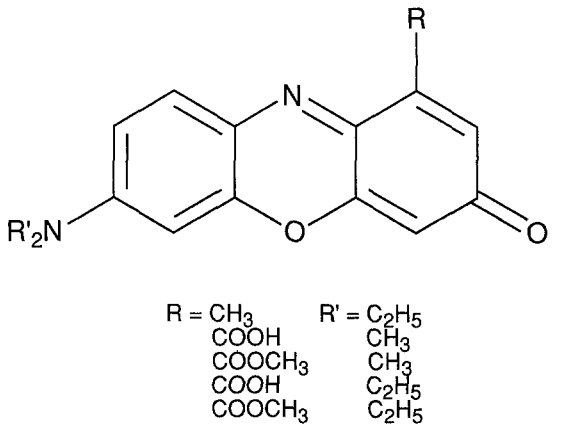

Fig. 2. Some oxazone derivatives previously synthesized from nitrosoaniline and resorcinol derivatives [6].

Fluorescence Measurements and Samples. Technical excitation and emission spectra were obtained on a Perkin Elmer LS-50 spectrofluorimeter using 3-ml quartz cuvettes under the conditions noted. Typically, the excitation and emission monochromator slit widths used were either 4.0 or 5.0 nm. No internal or external pass filters were used, and a constant photomultiplier voltage of 800 V was used for all samples. No spectral correction or modification of the spectra recorded was undertaken, with the exception of the normalization reported in Fig. 3. While the lack of spectral correction may indeed affect the intensities and shapes of spectra at higher wavelengths relative to those taken at lower wavelengths due to decreased red light photomultiplier sensitivity, this would not be expected to affect the relative fluorescence response reported under different conditions at particular individual wavelengths.

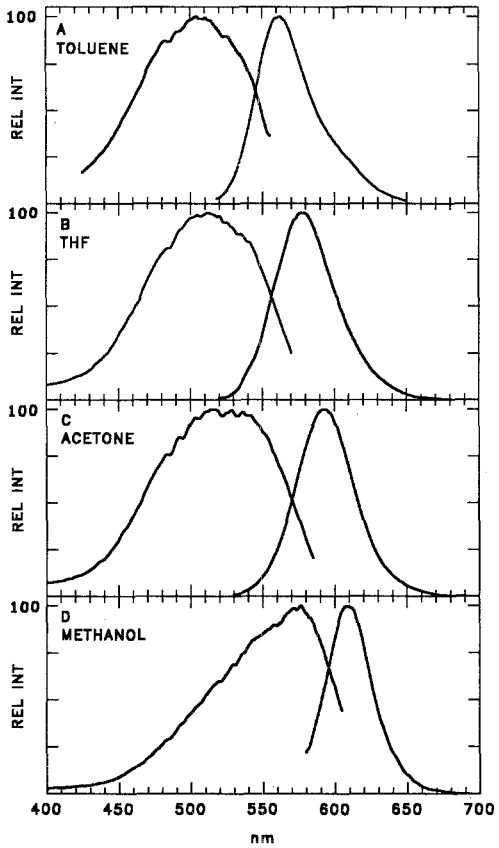

Fig. 3. Normalized excitation and emission spectra of 5 μ*M* 1-PDMPO solutions in various solvents. Excitation and emission slit widths were 6.0 nm for toluene and 4.0 nm for the remaining solvents. Increasing solvent polarity results in a shift of emission spectra to longer wavelengths.

Fluorescence lifetime and anisotropy data were obtained using instrumentation at the Center for Fluorescence Spectroscopy, University of Maryland—Baltimore. Multifrequency lifetime data were obtained using a Coherent Antares 76 Nd-YAG laser frequency doubled to 532 nm as the excitation source, with emission monitored through a magic-angle polarizer and a 600-nm band pass filter. The repetition rate of the laser was 76 MHz with a pulse width of 100 ps. Phase and modulation data were fitted to single exponentials using errors of 0.2° in phase and 0.005 in modulation, with chi-square values for the reported lifetimes ranging from 1.4 to 2.1. Double- and triple-exponential decay fits to these data did not result in substantial improvement in the chi-square values. An anisotropy excitation spectrum between 450 and 600 nm (620-nm emission) was obtained for 1-PDMPO (<1 μ*M*) in spectral-grade glycerol (Aldrich) at −15°C using a modified SLM 8000C fluorimeter in an "L" format.

Compound Synthesis, Purification, and Analytical Characterization. A typical synthesis and purification procedure went as follows: 0.500 g (3.33 mmol) of the nitrosoaniline dissolved in 40 ml of glacial acetic acid was combined with 0.600 g (3.33 mmol) of olivetol dissolved in 25 ml of glacial acetic acid in a 100-ml round-bottom flask. Boiling chips were added, and the mixture was gentled refluxed for 1 h. After cooling, the reaction mixture was placed in a 1000-ml beaker to which concentrated aqueous sodium carbonate was added. Initially, the basic solution was added dropwise, with increasingly greater volumes added as neutralization proceeded. The addition of base continued until a pH of 8 to 9 was reached, with the appearance of an insoluble green solid which tended to cling to the sides of the beaker. The contents of the beaker were then subjected to suction filtration, and the solid product stored in a desiccator prior to column chromatography. Thin-layer chromatography of this crude product on silica in a variety of developing solvents revealed several products as well as unreacted starting material.

Column chromatography resulting in purified 1-PDMPO was conducted on alumina as follows. Initially, 50 to 80 mg of the crude product was absorbed onto 2 to 4 g of alumina using a small portion of methylene chloride which was removed by evaporation. This alumina was then dry packed on the top of a glass column (ca. 200 × 10 mm) containing 90 g of alumina. Elution in a 1:1 (vol/vol) ethyl acetate/benzene eluant resulted in an early, slightly colored band followed shortly by a pink-purple band which readily fluoresced under a handheld UV lamp. This second brightly colored band was collected, dried under rotary evaporation, redissolved in methylene chloride, and finally, evaporated to dryness on a watch glass using a stream of nitrogen. Thin-layer chromatography of this band on silica, typically using a 70:30 hexane/acetone (v/v) eluant gave a single dark purple band (R_f, ca. 0.5). Mass spectral analysis of this product gave a molecular ion peak of 310 *m/e*, consistent with the proposed product. Proton nuclear magnetic resonance spectra taken in deuterated chloroform at 90 MHz gave chemical shifts and intensities consistent with 1-PDMPO.

RESULTS AND DISCUSSION

Fluorescence Spectra in Pure Solvents and Solvent Mixtures. Emission and excitation spectra of 1-PDMPO in several solvents and solvent mixtures are shown in

Figs. 3–5. The spectra shown in Fig. 3 have been normalized with respect to their maxima. Generally, 1-PDMPO showed increasing emission and excitation red shifts in pure solvents with increasing solvent polarity. However, fluorescence intensity was observed to decrease in both toluene and methanol at constant concentrations in comparison with tetrahydrofuran (THF), acetone, and chloroform (not shown), suggesting the possibility that particular solvent–solute interactions can significantly affect fluorescence. To investigate such effects further, 1-PDMPO fluorescence in hexane/acetone and DMSO/water mixtures was investigated. Poor solubility along with poor intensities made direct investigation of fluorescence spectra in pure water difficult.

Figure 4 shows the effect of changing hexane concentration on 1-PDMPO fluorescence in a series of hexane/acetone mixtures. As might be expected from the results in Fig. 3, decreasing polarity with increasing hexane content results in blue shifts to lower wavelengths

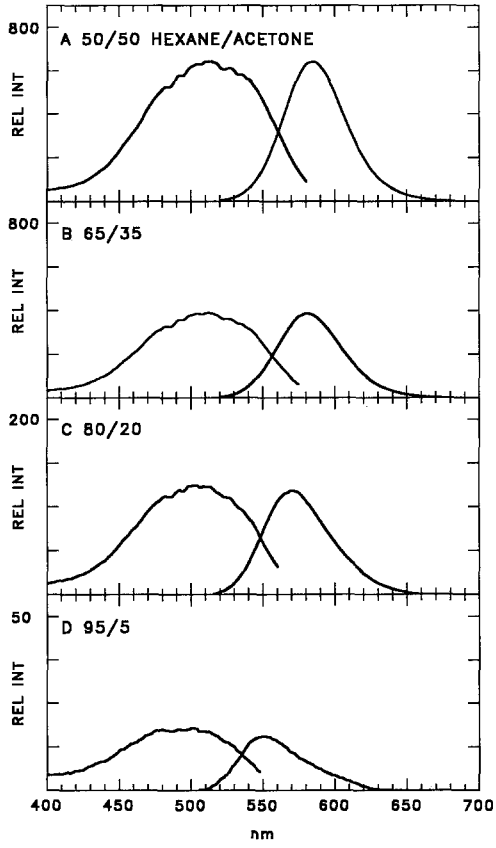

Fig. 4. Excitation and emission spectra of 5 μ*M* 1-PDMPO solutions in several hexane/acetone solvent mixtures. Hexane/acetone composition is reported as volume/volume ratio.

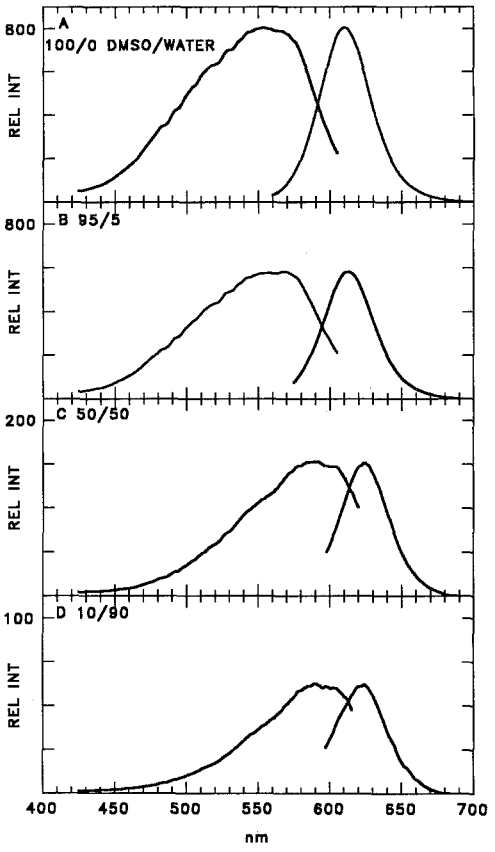

Fig. 5. Excitation and emission spectra of 5 μ*M* 1-PDMPO solutions in several DMSO/water solvent mixtures. DMSO/water composition is reported as volume/volume ratio.

in both the excitation and the emission spectra. Additionally, a significant decrease in fluorescence intensity is noticed with increasing hexane content, to the extent that only minimal fluorescence is detectable in 95/5 hexane/acetone mixtures. Figure 5 demonstrates qualitatively similar behavior with respect to intensity in DMSO/water mixtures with increasing water content. Consistent with the response to polarity shown in Fig. 3, both the excitation and the emission spectra shift to longer wavelengths with increasing water content, although the emission spectra in Fig. 5 show relatively smaller changes in the peak emission wavelength.

The observed responses of 1-PDMPO in hexane/acetone and DMSO/water mixtures differ to some extent from previously observed responses of the phenoxazone Nile Red under similar conditions. While Nile Red emission intensity also decreases with increasing water content in DMSO/water mixtures, much greater red shifts in emission peak wavelength are observed than with 1-

PDMPO [5]. On going from pure DMSO to 10/90 DMSO/water mixtures, for example, peak emission wavelengths vary from about 585 to about 660 nm (550-nm excitation) for Nile Red. An even greater difference is the response of the two probes in hexane-rich solvents; whereas 1-PDMPO fluorescence essentially cannot be detected in pure hexane, Nile Red at micromolar concentrations in hexane as well as heptane and cyclohexane demonstrates much stronger fluorescence emission [8].

Fluorescence Lifetimes and Anisotropy Spectrum. To characterize 1-PDMPO fluorescence further, single-exponential fluorescence decay lifetimes in several pure solvents and an anisotropy excitation spectrum in glycerol at −15°C were determined (Table I and Fig. 6). The decay lifetimes generally increase with increasing solvent polarity, with a decreased lifetime noted for the polar but protic solvent methanol. Trends in the decay lifetime data qualitatively match the fluorescence intensity variations noted above, with both toluene and methanol showing decreased lifetimes and intensities relative to solvents such as acetone and DMSO. The anisotropy excitation spectrum shows little structure and a monotonic increase toward 0.35 between 450 and 600 nm, suggesting that there is likely only a single emitting excited state whose dipole moment is roughly parallel to that of the ground state.

Table I. Monoexponential Fluorescence Decay Lifetimes for 1-PDMPO in Various Solvents[a]

Solvent	Dielectric constant[b]	Decay lifetime (ns)
Toluene	2.38	0.84
THF	7.58	1.85
Acetone	20.56	4.04
Methanol	32.66	2.17
DMSO	46.45	4.46

[a]Experimental conditions were as described (see text). Chi-square values for these fits ranged between 1.4 and 2.1 for assumed uncertainties in phase and modulation of 0.2° and 0.005, respectively. Trial bi- and triexponential fits did not result in an substantial improvement to chi-square values.

[b]Values at 25°C taken from Ref. 15.

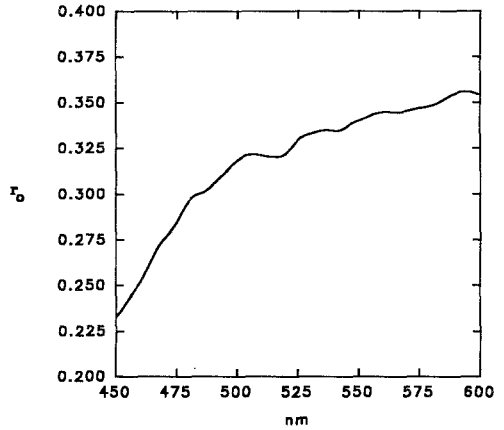

Fig. 6. Anisotropy excitation spectra of 1-PDMPO in glycerol at −15°C. Emission was monitored at 620 nm.

Increased fluorescent emission in aprotic polar solvents as opposed to aprotic nonpolar solvents is consistent with increased stabilization of an excited charge-transfer state for 1-PDMPO. Decreased stabilization and emission are observed from protic polar solvents such as alcohols which can hydrogen bond to the fluorophore. Comparison with available lifetime data for Nile Red in nonpolar, polar, and protic solvents, however, suggests that the decreased conjugation in 1-PDMPO results in decreased excited state stability. Generally, measured decay lifetimes in a variety of solvents appear to be greater for Nile Red, ranging from roughly 3 ns in hexane, cyclohexane, and heptane, to 4.5–4.8 ns in toluene and THF, 5.4–5.5 ns in acetone and acetonitrile, and decreasing again to roughly 3 ns in short-chain alcohols [8,9]. While the effects of structural modifications to the oxazone backbone require further investigation, this observation could have implications for the synthetic design of long-wavelength fluorescent probes for biological imaging.

Acid–Base Response. Viewed from another perspective, 1-PDMPO can be thought of as a lipophilic derivative of the classic dye resorcin blue (MLB) [10]. In the past, this dye has been used as an acid–base indicator; this, as well as the response of 1-PDMPO to protic solvents, suggested that 1-PDMPO acid/base fluorescence response also be investigated. Observed changes in emission spectra in 1 μM 1-PDMPO solutions in the presence of HCl and KOH in methanol are compared to emission spectra in pure methanol in Fig. 7A. The methanolic HCl and KOH solutions were prepared using concentrated reagent HCl and reagent KOH, respectively. 1-PDMPO showed a rapid decrease in fluorescence emission intensity at low HCl concentrations in methanol, while less of an effect on fluorescence intensity was noted at higher base concentrations. Figure 7B shows how microliter additions of a 2.5×10^{-3} M methanolic HCl solution to 3 ml of a 1 μM methanolic solution of 1-PDMPO decreased fluorescence emission intensity. Effectively, this represents a titration of a basic functionality on 1-PDMPO. Earlier work on resorcin blue (MLB) suggests that its carbonyl oxygen undergoes pro-

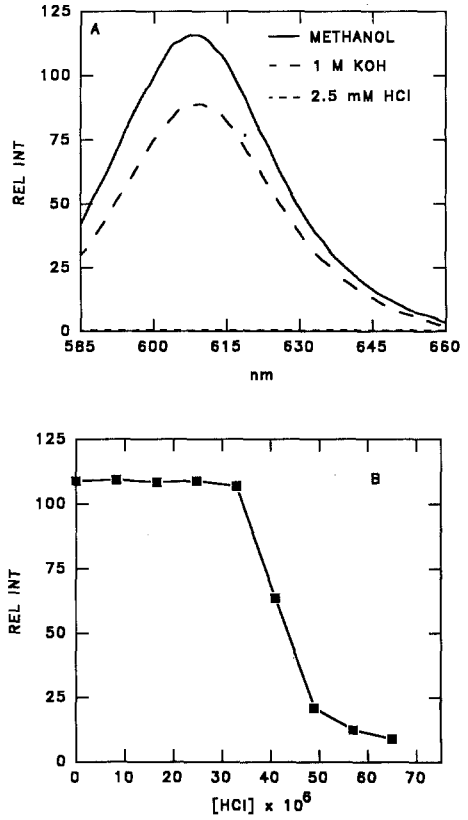

Fig. 7. (A) Emission spectra of 1 μ*M* 1-PDMPO solutions in methanol and methanolic HCl and KOH solvents. Excitation was at 575 nm. (B) Emission response of a 1 μ*M* 1-PDMPO methanol solution (total volume, 3 ml) at 610 nm to successive 10-μl additions of 2.5×10^{-3} *M* methanolic HCl. The sample was stirred and allowed to equilibrate prior to each measurement.

tonation with a pK_a value of 4 [4]. Protonation of 1-PDMPO in the ground state may prevent formation of a fluorescent excited state or provide nonfluorescent relaxation pathways once excited states are formed. The observed sensitivity to acid as opposed to base can also be rationalized in terms of charge transfer, in that a hydroxide ion would be less likely to interact with lone pairs or a proton-accepting carbonyl involved in a charge-transfer process.

Aqueous Sodium Dodecyl Sulfate Solutions. Recently, Nile Red has been used to investigate the properties of proteins in sodium dodecyl sulfate (SDS) suspensions [11]. The fluorescence sensitivity of Nile Red to aqueous environments has also been used to characterize detergent physical properties [12]. Given this use, it seemed appropriate also to measure the 1-PDMPO fluorescence response in aqueous SDS solutions above the critical micellar concentration. Emission and excitation spectra of 1-PDMPO at 1 μ*M* concentration in 50 m*M* aqueous SDS solutions are shown in Fig. 8A. The solutions were labeled by the injection of 6 μl of a 5×10^{-4} *M* 1-PDMPO solution in acetone into 3 ml of SDS solution. Comparison of Figs. 8A and D (representing the emission spectra in methanol) suggested that in an SDS micelle, 1-PDMPO may be subject to a similar polarity. From these data it appears that 1-PDMPO and structural analogues should have utility in the characterization of micellar physical properties.

Egg Phosphatidylcholine and Bovine Serum Albumin (BSA). Solvatochromic fluorophores are also of in-

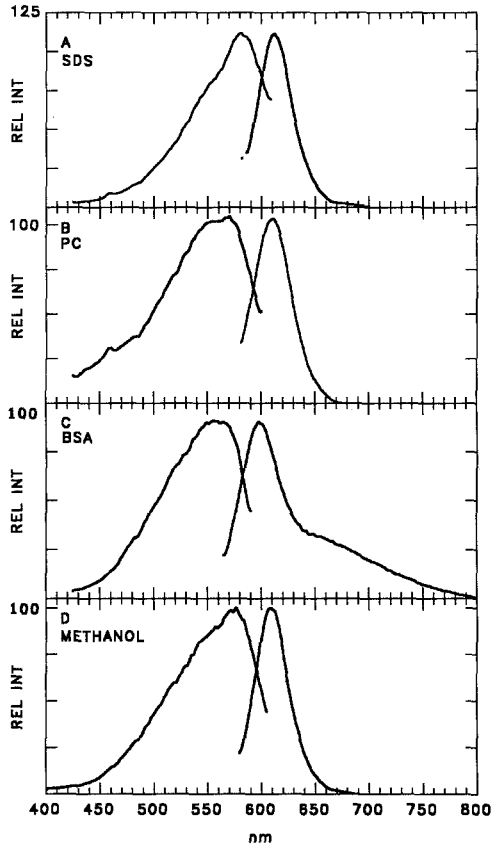

Fig. 8. (A) Excitation and emission spectra of 1-PDMPO-labeled aqueous 50 m*M* SDS solutions. Total 1-PDMPO concentration was 1 μ*M*. (B) Excitation and emission spectra of 1-PDMPO-labeled egg phosphatidylcholine (PC) extruded vesicles. Total 1-PDMPO concentration, 1 μM; egg phosphatidylcholine concentration, 200 μ*M*. (C) Excitation and emission spectra of 1-PDMPO-labeled fatty acid-free bovine serum albumin (BSA) suspension. Total 1-PDMPO concentration, 1 μ*M*; BSA concentration, 1 mg/ml. (D) Comparable excitation and emission spectra of 1-PDMPO in methanol.

terest in the study of protein and membrane structure, in that their fluorescence response gives information on the environment of the fluorophore. Data from Nile Red binding in tubulin, for example, have been used to construct a model predicting two binding sites on tubulin dimers with differing polarities [13]. Here, the 1-PDMPO fluorescence response in extruded egg phosphatidylcholine vesicles and BSA suspensions was investigated. Emission and excitation spectra for 1-PDMPO-labeled extruded egg phosphatidylcholine vesicles and a 1-PDMPO-labeled suspension of essentially fatty acid-free BSA (Sigma) are shown in Figs. 8B and C. Labeling was carried out at 37°C over a period of 15–20 min after the addition of 6 μl of a 5×10^{-4} *M* 1-PDMPO solution in acetone to 3 ml of each sample in 10 m*M* Tris–HCl, 150 m*M* NaCl buffer (pH 7.4). The total added 1-PDMPO concentration was 1 μ*M*, with the egg phosphatidylcholine vesicles at a concentration of 200 μ*M* and BSA at 1 mg/ml, respectively.

As for SDS micelles, 1-PDMPO in these two systems demonstrates an emission peak suggestive of a fluorophore environment whose polarity is roughly comparable to that of pure methanol. However, a large red-shifted shoulder observed is also observed in emission in the BSA suspension, extending nearly to 800 nm. The presence of this shoulder seems to suggest that in BSA suspensions, 1-PDMPO experiences markedly different excited-state relaxation processes in comparison to phospholipids. Given the roughly 1/14 molar 1-PDMPO/protein ratio in this system, it also seems reasonable that this response reflects different types of 1-PDMPO binding or association with the protein, rather than self-association or interaction. With detection or lifetime decay analysis at longer wavelengths, this observation might be conceivably be exploited in the study of protein binding or protein–lipid interfacial properties.

CONCLUSIONS

Initial characterization of the fluorescence response of 1-PDMPO suggests that its solvatochromic properties, as well as its sensitivity to acid, could make it a useful fluorescence probe in the mold of such compounds as PRODAN, Nile Red, and lipophilic coumarin derivatives. Structural analogues of this compound can easily be envisioned, which may offer possibilities for better characterization of detergent, membrane, and protein systems. Synthetic use of resorcinol derivatives with longer acyl chains and alternative ring positions, for example, may offer the opportunity of improved fluorophore localization in biological or biomimetic structures. Derivatization reagents for protein studies utilizing this oxazone backbone are also a possibility, as has recently been reported for Nile Red [14]. The excitation spectra reported suggest that this oxazone backbone is also a good candidate for studies utilizing green laser excitation sources, although comparison of 1-PDMPO decay lifetime data in pure solvents with those for Nile Red suggests that the extra phenyl ring in the latter compound may offer advantages for biological imaging. Given the current interest in long-wavelength fluorescent dyes and probes, further investigation of the properties of fluorescent oxazone and phenoxazone compounds appears warranted.

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