JOURNAL OF PHYSICAL ORGANIC CHEMISTRY
J. Phys. Org. Chem. 2007; **20:** 624–629
Published online in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/poc.1222

Ethenyl indoles as neutral hydrophobic fluorescence probes

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Received 26 December 2005; revised 3 September 2006; accepted 15 March 2007

ABSTRACT: 3-(4-Nitrophenylethenyl-*E*)-NH-indole (**1**), 3-(4-nitrophenyl ethenyl-*E*)-*N*-acetylindole (**2**), and 3-(4-nitrophenylethenyl-*E*)-*N*-benzenesulfonylindole (**3**) are relatively less fluorescent in organic solvents, with fluorescence quantum yield (Φ_f) in the range of 0.002 to 0.066 depending on the solvent polarity. However, in bovine serum albumin (BSA)-phosphate buffer, the fluorescence of these compounds gets drastically enhanced with Φ_f in the range of 0.21 to 0.67, depending on the substituent on the indolic nitrogen atom. Additionally, linear increase in the fluorescence intensity of **2** and **3** occurs on increasing the BSA concentration. These fluorescence properties together with the neutral, hydrophobic nature of these compounds make these fluorophores good fluorescence probe for studying the micropolarity of proteins like BSA and in general the ligand–protein interactions. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: serum albumin; ethenyl indoles; fluorescence; binding constant; fluorescence quenching

INTRODUCTION

Extrinsic fluorescence probes are used extensively for probing ligand–biomolecule binding and solubilization sites in organized assemblies.[1–6] However, the information that is obtained from these probes is also due to secondary Columbic interactions that these probes undergo with the host system. This is because of self-ionic behavior of the probes. Probing studies with neutral probes are ideal but limited.[7] Development of neutral probes bearing greater sensitivity to their local environment and greater ability to get anchored at a known location within the complex host system is, therefore, desirable.

Donor–acceptor diphenylpolyenes capable of fluorescing from their charge-transfer excited states have been used to study the microenvironment of micelles and proteins.[8–11]. It has recently been observed that the donor–acceptor diphenylpolyenes containing indole unit (e.g. ethenyl indoles) not only show solvatochromic fluorescence from their charge-transfer excited state but also fluoresce more efficiently as compared to the diphenylpolyenes devoid of an indole moiety.[12,13] This promoted us to examine the fluorescence probe properties of ethenyl indoles in a protein environment.

In this paper, we report fluorescence properties of ethenyl indoles **1–3** (Figure 1) in aqueous buffer and in bovine serum albumin (BSA), a well-known protein responsible for transport of a variety of ligands[14]. A comparison of the present results with the fluorescence properties of some well-known compounds reveals that these indolic compounds can be used as a fluorescence probe for studying the microenvironment of biomolecular and organized systems.

EXPERIMENTAL

3-Formylindole, BSA (fraction V) and other chemicals were purchased from SRL, Mumbai, India. UV grade solvents for spectroscopic studies and AR grade solvents for synthetic purposes were either from Spectrochem or E. Merck (India), Mumbai, India. Petroleum ether (60–80°C fraction) procured from local suppliers was distilled prior to its use. Thin layer and column chromatographic analyses were done using silica gel G (Merck). The absorption spectra were measured using a Shimadzu UV–160A spectrophotometer. FTIR spectra in KBr discs were recorded on Impact Nicolet-400 spectrophotometer. Melting points were determined on a Veego melting point apparatus. The ^{1}H NMR spectra in $CDCl_3$ using TMS as internal standard were recorded on Varian VXR 300 MHz FTNMR instrument. Mass spectra were recorded on QT of Micro (YA-105), Micro mass (waters) through electrospray ionization. CHN analyses

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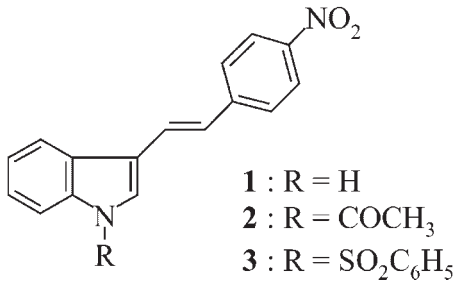

Figure 1. Structure of compounds **1–3**

were performed on Theoquest CE instrument 1112 series CHNS autoanalyzer. The fluorescence spectra were recorded on Perkin–Elmer LS-55 spectrofluorimeter. The fluorescence emission spectra were recorded by exciting at the absorption maximum ($\lambda_{abs\ max}$) of the respective compounds. Fluorescence quantum yields (Φ_f) were determined against rhodamine B in ethanol ($\Phi_{f\ ref} = 0.69$)[15] as standard. For all the electronic spectroscopic (absorption, fluorescence, excitation, and emission) studies in solvent, 2.0×10^{-5} M solutions of the respective compounds were used. For all the protein studies, phosphate buffer, pH = 7.4 was used throughout the experiment. A varied concentration of BSA (from 1×10^{-6} to 1×10^{-4} M) was added to either 2×10^{-6} M (for **1**) or 2×10^{-5} M (for **2** and **3**) in phosphate buffer solution. For quenching studies, a varied range of compound concentrations (2×10^{-6} to 2×10^{-5} M) were added to a phosphate buffer solution (pH = 7.4) of BSA (1×10^{-6} M).

Compounds **1–3** were prepared in a manner similar to our previously reported procedures.[12,13] All the three compounds exhibited physico–chemical data characteristics of their structure and identical to the data reported earlier.

RESULTS AND DISCUSSION

The absorption and fluorescence data of **1–3** are shown in Table 1. The absorption maximum ($\lambda_{abs\ max}$) of **1–3** is similar in solvents like methanol and acetonitrile. However, a moderate red-shift in $\lambda_{abs\ max}$ is observed in the phosphate buffer. On the other hand, the fluorescence emission maximum ($\lambda_{em\ max}$) is highly red-shifted in polar aprotic solvents like acetonitrile. Thus, the $\lambda_{em\ max}$ is red-shifted by 131 nm, 179 nm and 73 nm for **1–3**, respectively when the solvent changed from nonpolar heptane to polar acetonitrile. Compounds **1–3** exhibit low fluorescence quantum yield in phosphate buffer (Φ_f **1**: 0.024, **2**: 0.003, **3**: 0.003). Also, compounds **2** and **3** show inefficient fluorescence (Φ_f **2**: 0.008, **3**: 0.004) in nonpolar solvent heptane, whereas, compound **1** is moderately fluorescent (Φ_f: 0.051).

Upon binding to BSA, the fluorescence efficiency of these compounds increases drastically. Thus, Φ_f of BSA-probe complex increases 30 times (for **1**) and 100 times (for **2** and **3**), as compared to the unbound probe in buffer. Similarly, Φ_f of BSA-probe complex increases 10 times (for **1**) and 50 times (for **2** and **3**), as compared to in nonpolar solvent. The fluorescence spectra on increasing concentration of BSA are shown in Figure 2. While, interaction of **2** and **3** with BSA results in a blue-shift of 84 nm in their $\lambda_{em\ max}$ (i.e. 537/540 nm to 453/456 nm), the $\lambda_{em\ max}$ of **1** is insensitive to the concentration of BSA (i.e. 552 nm). Interestingly, compound **2** shows dual fluorescence on increasing concentration BSA. Thus, the $\lambda_{em\ max}$ of **2** in phosphate buffer at 537 nm disappears on increasing the BSA concentration, and a new fluorescence emission peak appears at 453 nm. Compounds **2** and **3** are

Table 1. Absorption maximum ($\lambda_{abs\ max}$), fluorescence emission maximum ($\lambda_{em\ max}$), excitation maximum ($\lambda_{ex\ max}$), and fluorescence quantum yield (Φ_f) of **1–3** in various solvents and BSA

Compound	Solvent	$\lambda_{abs\ max}$ (nm)	$\lambda_{em\ max}$ (nm)	$\lambda_{ex\ max}$ (nm)	$\Phi_f \pm 0.0003$
1	Heptane	393	511	402	0.051
	Methanol	413	567	416	0.003
	Acetonitrile	410	642	405	0.009
	Buffer sol[n]	418	552	411	0.024
	BSA	410	552	410	0.67
2	Heptane	363	408, 424	363	0.008
	Methanol	374	424, 563	375	0.002
	Acetonitrile	377	428, 603	374	0.045
	Buffer sol[n]	381	537[a]	370	0.003
	BSA	376	453	375	0.31
3	Heptane	357	403, 424, 520	356	0.004
	Methanol	367	417, 546	372	0.002
	Acetonitrile	369	593	369	0.066
	Buffer sol[n]	378	540[a]	371	0.003
	BSA	370	456	371	0.21

[a] Negligible fluorescence.

DOI: 10.1002/poc

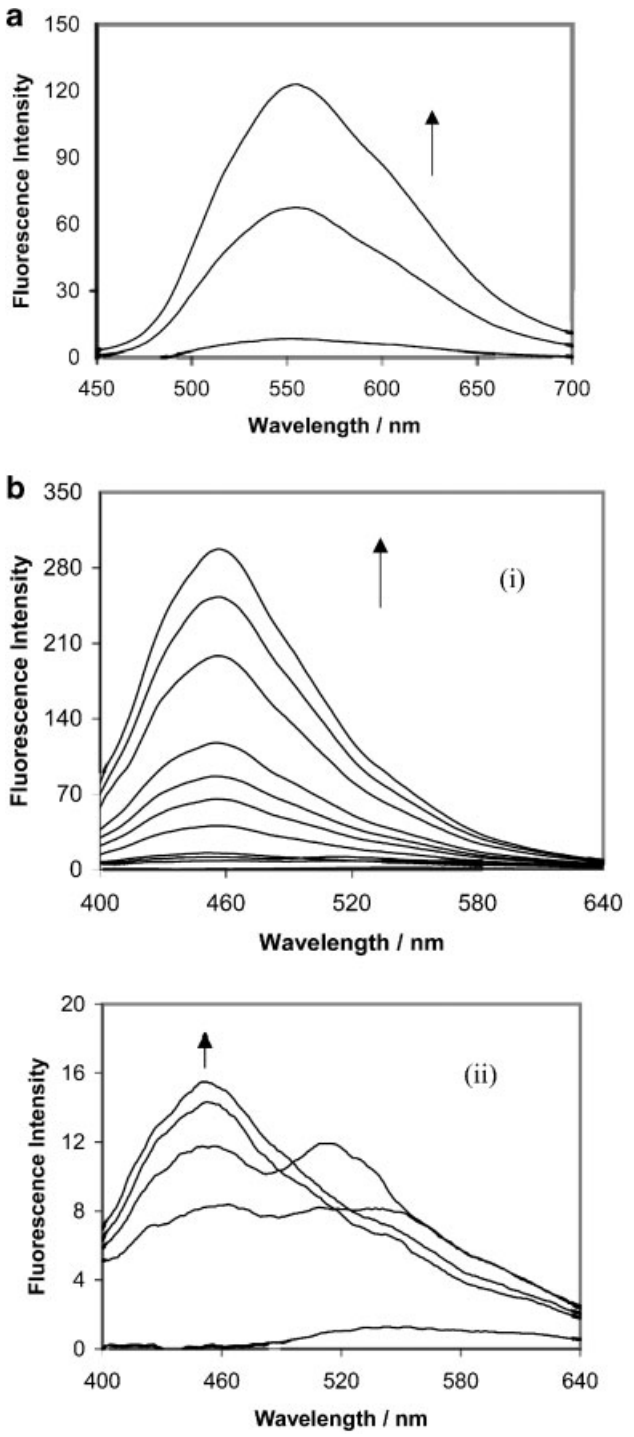

Figure 2. (a) Fluorescence spectra of **1** on increasing concentration of BSA. (0, 1, 2×10^{-6} M) and (b) Fluorescence spectra of **2** on increasing concentration of BSA (i) [BSA]: (0, 5, 10, 30, 50, 70, 90, 100, 200, 300, 400×10^{-6} M) and (ii) [BSA]: (0, 5, 10, 30, 50×10^{-6} M)

known to show dual fluorescence in organic solvents.[12,13] The $\lambda_{em\ max}$ of <450 nm is attributed to the locally excited (LE) state, while the $\lambda_{em\ max} \sim 500$ nm is believed to arise from a highly polar, conformationally relaxed intramolecular charge transfer (CRICT) excited state. Plausible structures of LE and CRICT species are shown in Figure 3.

The present results suggest that the nonpolar, LE states of **2** and **3** are predominantly formed in BSA. However, in case of **1**, the polar, CRICT excited state is formed in the protein matrix. These results also indicate that while **1** binds to the hydrophilic pocket of BSA, fluorophores **2** and **3** having hydrogen bonding capable substituents like —CO (acetyl) and —SO (sulfonyl) interact with the hydrophobic pocket of BSA.

The BSA-fluorophore binding constant (K) was calculated using[16]

$$(\Phi/\Phi_a - 1)^{-1} = (\Phi_b/\Phi_a - 1)^{-1}(1 + 1/KC) \quad (1)$$

where, Φ is the fluorescence quantum yield of compound in the presence of BSA, Φ_a is the fluorescence quantum yield of compound in absence of BSA, Φ_b is the fluorescence quantum yield of fluorophore–BSA complex, C is the concentration of BSA, and K is the binding constant. A high value of $K \sim 10^7\,M^{-1}$ is found for **1** which is 10–50 times more than the binding constant obtained for **2** and **3** ($\sim 2.0 \times 10^5\,M^{-1} - 1.0 \times 10^6\,M^{-1}$) having blocked indole —NH (Table 2). Thus, —NH group of indole plays important role for binding to BSA. Interestingly, fluorescence intensity of **2** and **3** bearing the hydrophobic substituent is linearly increased up to 100 μM concentration of BSA (Fig. 4). Thus, **2** and **3** can be used for the quantification of BSA as well.

The interaction of such a probe with BSA is also monitored by tryptophan fluorescence quenching of BSA with increasing probe concentration and using the Stern–Volmer equation[17] (Eqn (2)), and for this the tryptophan fluorescence was monitored at 346 nm with excitation of BSA at 280 nm, where the ethenyl indoles do not absorb significantly (Figs 5 and 6).

$$F_0/F - 1 = K_{sv}[Q] = k_q\tau_0[Q] \quad (2)$$

where, F_0 is the fluorescence intensity of protein emission in the absence of quencher, F is the fluorescence intensity of protein emission in the presence of quencher, K_{sv} is the Stern–Volmer quenching constant, $[Q]$ is the quencher concentration, k_q is the bimolecular quenching rate constant, and τ_0 is the fluorescence life time of the protein in the absence of quencher (6.1 ns, reported value for BSA).[18]

The Stern–Volmer quenching constant (K_{sv}) is two times greater for **2** and **3** as compared to **1**. The Stern–Volmer plot linearly increases which indicates that one of the tryptophan residues exposes to the quencher. It is found that at the concentration of 2.38×10^{-5} M (for **1**), 1.41×10^{-5} M (for **2**) and 1.24×10^{-5} M (for **3**), 50% of tryptophan fluorescence intensity is quenched by the quencher **1–3**. Further, the bimolecular quenching rate constant (k_q) is in the order of $5 \times 10^{12}\,M^{-1}s^{-1}$ (for **1**) and $1 \times 10^{13}\,M^{-1}s^{-1}$ (for **2** and **3**). Thus, in the present case k_q is 500–1000 times higher than the expected rate constant for a diffusion-controlled bimolecular process (in order

DOI: 10.1002/poc

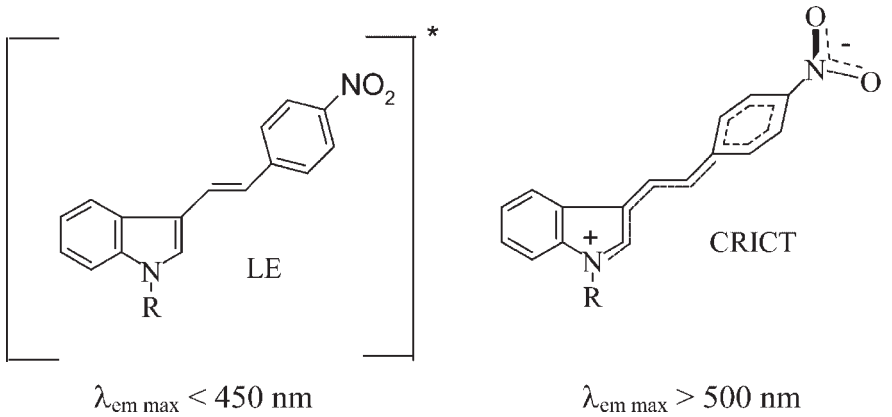

Figure 3. Plausible structure of nonpolar LE state and polar CRICT excited state of ethenyl indole

Table 2. Binding constant (K), Stern–Volmer quenching constant (K_{sv}), and bimolecular quenching constant (k_q) for **1–3** in BSA

Compound	K (10^6) M^{-1}	R^2	K_{sv} (10^4) M^{-1}	R^2	k_q (10^{13}) $M^{-1}s^{-1}$
1	13.90	0.92	4.19	0.95	0.686
2	1.260	0.92	7.09	0.84	1.162
3	0.319	0.98	8.06	0.88	1.321

R^2: The linearity.

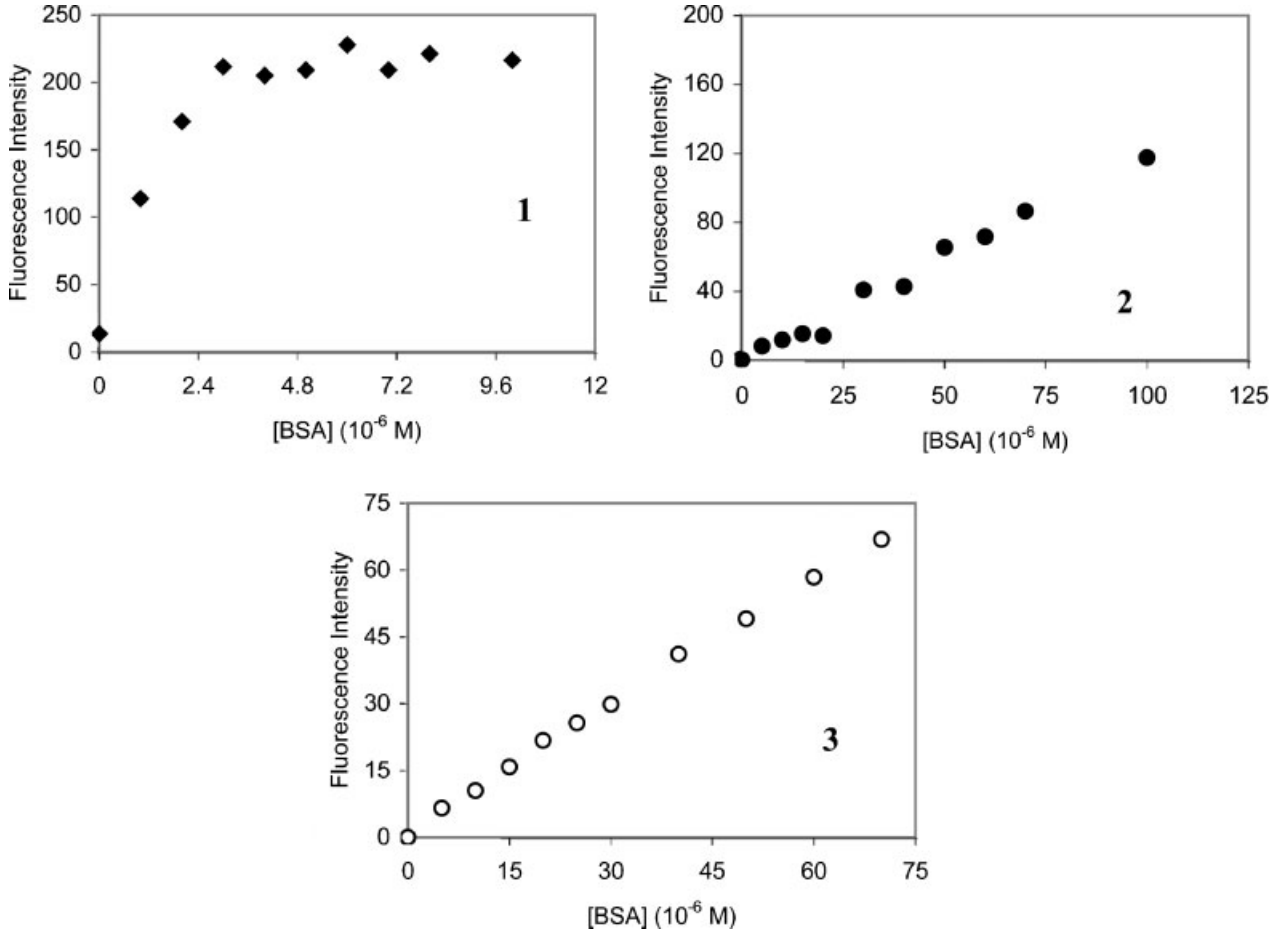

Figure 4. Plot of fluorescence intensity *vs*. BSA concentration for **1**(◆), **2**(●), and **3**(○)

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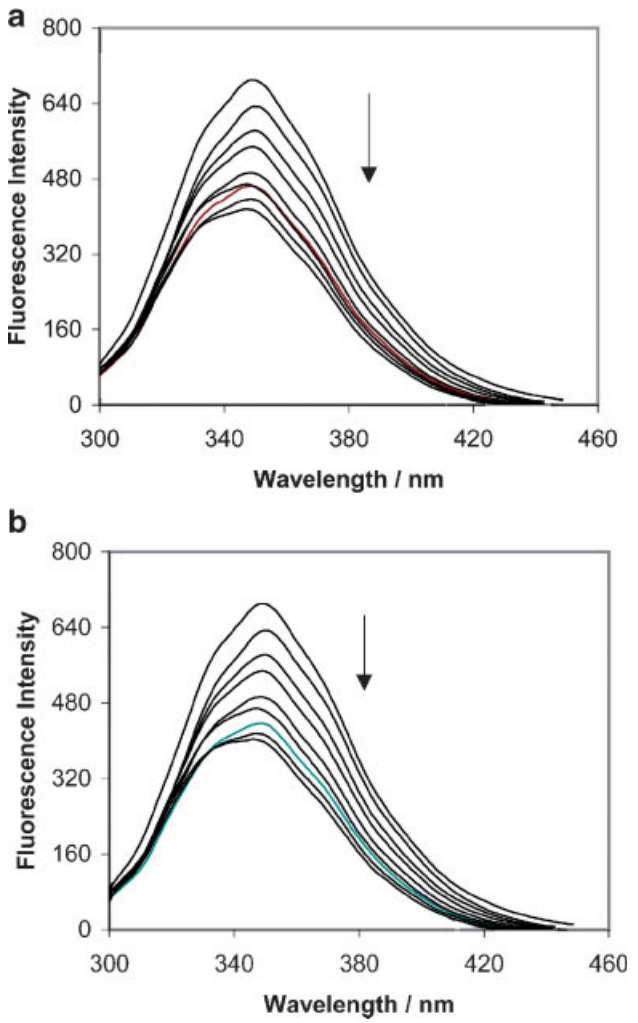

Figure 5. (a) Tryptophan fluorescence quenching spectra of BSA, in the presence of increasing concentration of quencher **1** and (b) Tryptophan fluorescence quenching spectra of BSA in the presence of increasing concentration of quencher **2**.

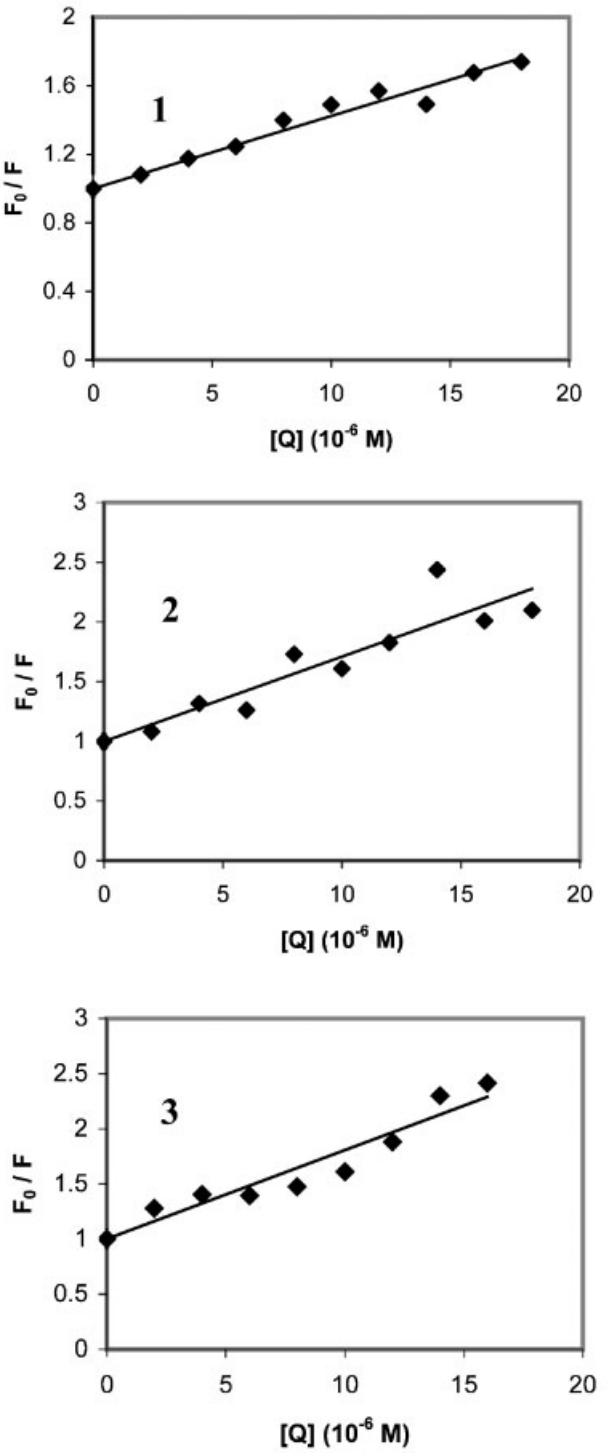

Figure 6. Stern–Volmer tryptophan fluorescence quenching plots for BSA in the presence of quencher **1–3**

$1 \times 10^{10}\,M^{-1}s^{-1}$).[16] This can be due to the strong interaction of tryptophan residue with the probe molecules. As k_q is in the order of $\mathbf{3} > \mathbf{2} > \mathbf{1}$, it can be suggested that probe **3** bearing $SO_2C_6H_5$ group is in close proximity to tryptophan residue as compared to probe **1** having free indole —NH.

The fluorescence properties of the indolic ethenyl compounds are compared with the known fluoresecent probe such as 1-(anilino)naphthalene-8-sulfonate (ANS)[1,19], 6-propionyl-2-(*N,N*-dimethyl-amino)naphthalene (PRODAN)[7,19], and 11-(dansylamino) undecanoic acid (DAUDA)[20]. The parameters like $\lambda_{em\ max}$ in phosphate buffer and in protein matrix and the binding constant (*K*) are summarized in Table 3. The $\lambda_{em\ max}$ of known probes like ANS, PRODAN and DAUDA as well as probes **1–3** gets blue-shifted upon binding to BSA. However, as compared to these known probes, the magnitude of this blue-shift is larger (84 nm) for **2** and **3**. This indicates towards a strong interaction between the indolic ethenyl probes and BSA. A binding constant of $6.2 \times 10^6\,M^{-1}$ (for ANS), $1.0 \times 10^6\,M^{-1}$ (for PRODAN), $1.2 \times 10^6\,M^{-1}$ (for DAUDA), in the phosphate buffer have been

DOI: 10.1002/poc

Table 3. Fluorescence ($\lambda_{em\ max}$), binding constant (K), and blue-shifted emission maximum ($\Delta\lambda$) of few studies reported fluorescence probes and indolic ethenyl **1–3** in BSA and phosphate buffer solution

Probe	$\lambda_{em\ max}$ (nm)		$\Delta\lambda$ (nm)	K (M^{-1})	Fluorescence enhancement[a]
	Phosphate buffer soln	In BSA			
ANS	509	471	38	6.2×10^6	15
PRODAN	511	469	42	1.0×10^6	10
DAUDA	536	495	41	1.2×10^6	100
1	552	552	0	1.3×10^7	20
2	537	453	84	1.2×10^6	100
3	540	456	84	3.2×10^5	70

[a] Fluorescence enhancement in BSA as compared to phosphate buffer.

reported. As compared to these probes, the binding constants of $13.9 \times 10^6\,M^{-1}$ (for **1**), $1.2 \times 10^6\,M^{-1}$ (for **2**) and $3.2 \times 10^5\,M^{-1}$ (for **3**) are observed for indolic ethenes. This shows that the binding behavior of indolic ethenes is similar to that of ANS, PRODAN, and DAUDA. Further, as compared to **1**, probes **2,** and **3** are weakly bound to the BSA matrix.

All these probes show 10–100 times enhancement in their fluorescence intensity upon binding to BSA. In the case of indolic ethenes **2** and **3**, the fluorescence intensity increases linearly with respect to BSA concentration, making theses fluorophores as useful probes for protein quantification. The present indolic ethenes are neutral as these do not have permanent charge. Hence, the unnecessary Coulombic interaction between the fluorophore and its surrounding medium (charged amino residues in case of a protein) is negligible. Therefore, these fluorophores can be used for studying the protein–ligand interaction.

CONCLUSIONS

In summary, ethenyl indoles show efficient fluorescence upon binding to BSA and the fluorescence intensity of ethenyl indoles **2** and **3** increases linearly with respect to BSA concentration. The indole —NH group plays important role in binding to BSA. The ethenyl indoles are neutral fluorescence probe, which can be used for studying the protein–ligand interaction and their binding behavior.

Acknowledgements

Financial support to a project from the Board of Research in Nuclear Sciences (DAE, Government of India) and a research fellowship to P.K.H. from the University Grants Commission, New Delhi, India is gratefully acknowledged.

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DOI: 10.1002/poc