Novel 7-(dimethylamino)fluorene-based fluorescent probes and their binding to human serum albumin† ‡

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A novel solvatochromic fluorescent molecule, 9,9-dibutyl-7-(dimethylamino)-2-fluorenesulfonate **2** was synthesized from 2-nitrofluorene in moderate yield. The fluorescence spectra of **2** and 7-(dimethylamino)-2-fluorenesulfonate **1** shift to shorter wavelengths as the polarity of the medium decreases. Both **1** and **2** bind to hydrophobic sites of human serum albumin (HSA). The apparent binding constants were determined by fluorescence titration to be 0.37×10^6 M^{-1} for **1** and 2.2×10^6 M^{-1} for **2**. The energy of the Trp-214 fluorescence of HSA is transferred to the HSA-bound fluorophores with near 100% efficiency. The covalent bonding of acrylodan (AC) to Cys-34 has little effect on the binding affinity of **2** to HSA or fluorescent behavior of HSA-bound **2**. Bound **2** also has little effect on the fluorescence of AC, but **2**→AC and Trp-214→**2**→AC resonance energy transfers were observed. Competitive binding between the fluorene compounds and other ligands such as 1-anilino-8-naphthalenesulfonate, aspirin, *S*-(+)-ibuprofen and phenylbutazone were also studied fluorometrically. The results indicated that the primary binding site of **2** to HSA is site II in domain IIIA, whereas **1** binds to site I in domain IIA, but a different region from the phenylbutazone binding site. Because of its large molar absorptivity, strong fluorescence, sensitivity to its environment, and high binding constant to HSA, **2** can be used successfully in the study of proteins and their binding properties.

Introduction

Fluorescent molecules whose spectral properties are exquisitely sensitive to their environments have been widely used as probes for the study of membrane microenvironments and hydrophobic domains on proteins, as well as the dynamics and conformational changes of macromolecules.[1,2] In addition to the environmental sensitivity of the fluorescent properties, a large molar absorptivity and fluorescence quantum yield are desirable characteristics for a highly sensitive fluorescent probe. Moderate solubility in aqueous media and high binding affinity for the target are also requisite for applications with biological molecules.

Human serum albumin (HSA) is the most abundant protein in the circulatory system, and its principal function is to transport a variety of bioactive molecules such as fatty acids, steroid hormones, vitamins and numerous pharmaceuticals. HSA also contributes significantly to colloid osmotic pressure.[3,4] HSA contains 585-amino acids with three homologous α-helical domains (I-III) and has a single tryptophan (Trp-214) residue.[4-8] Hydrophobic or amphiphilic ligands for HSA bind mostly to one of the two principal binding sites, located in the subdomain IIA (site I) and IIIA (site II) (Fig. 1).[3,5-11] These binding sites are often selective, however, binding to multiple sites on HSA has also been observed.[5,6,8-16] The HSA-binding affinity of a compound is the key factor for determining the concentration of its unbound form in the plasma. Thus the binding affinity of a drug to HSA affects the distribution, pharmacokinetics, toxicity and rate of excretion of the drug.[3,8] Therefore, information on the binding affinity of a drug to HSA is particularly useful for the identification of potential *in vivo* pharmaceutical problems.[11,17]

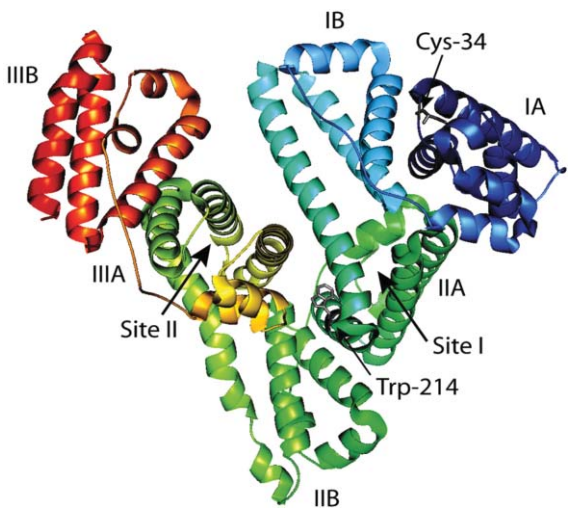

Fig. 1 Crystal structure of human serum albumin and the location of major binding sites. The positions of Trp-214 and Cys-34 are shown. The structure was obtained from a protein data bank (1ha2).

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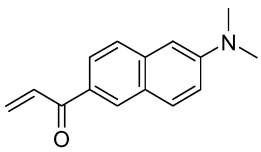
‡ Electronic supplementary information (ESI) available: Absorption, fluorescence and excitation spectra of **1** and **1**/HSA mixture; fluorescence spectra of **1**/HSA mixture in the presence of aspirin. See DOI: 10.1039/b911605b

A variety of naphthalene-based fluorescent probes such as 5-(dimethylamino)-1-naphthalenesulfonate,[18] 1-anilino-8-naphthalenesulfonate (ANS),[15,16,19,20] 6-acryloyl-2-dimethylaminonaphthalene (acrylodan),[21-26] 6-propionyl-2-dimethylaminonaphthalene (prodan),[22,26-28] 6-propionyl-2-methoxynaphthalene (promen),[22,29] orange II,[30] dansyl derivatives,[9,14,31-33] and 1-hydroxy-2-acetonaphthone[34] have been used for fluorescent studies of HSA: acrylodan binds to Cys-34 covalently, while others bind non-covalently to HSA. Other fluorescent molecules, *e.g.* warfarin[9,11,14,31,33,35,36] and 2-(6-diethylaminobenzo[*b*]furan-2-yl)-3-hydroxychromone[36] have also been used for this role. Using these probes, the characteristics of ligand binding and binding sites of HSA, competitive binding of other ligands, and the spatial relationship between Trp-214 and the probe-binding sites have been investigated. Also the fluorescent probes have been utilized for the investigation of unfolding processes[20,21,26,28,32] and dynamics[25,30] of HSA.

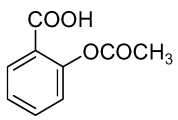
It has been reported that fluorescent properties of aminofluorenes are sensitive to solvent environments, in a similar way to aminonaphthalenes.[37-39] This, together with the large molar absorptivity, high fluorescence quantum yield,[38-40] and high binding affinity of 2-(dimethylamino)fluorenes to β-amyloid,[41] suggested that aminofluorene derivatives might be novel and sensitive fluorescent probes for proteins and biomimetic systems. In a previous paper, we reported a novel water-soluble aminofluorene derivative, 7-(dimethylamino)-2-fluorenesulfonate **1** and the effects of solvent polarity on its absorption and fluorescence behavior.[39] Here we report the synthesis of another water-soluble fluorene derivative, 9,9-dibutyl-7-(dimethylamino)-2-fluorenesulfonate **2**, its solvent-dependent fluorescent properties, and a comparative HSA binding study of **1** and **2**. We demonstrate that the fluorene compounds can be used as highly sensitive fluorescent probes for studies of biological and bio-mimetic systems.

Results and discussion

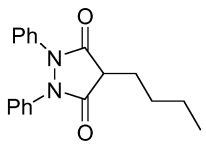
The ammonium salt of **1** is slightly soluble in water and in polar organic solvents, but practically insoluble in non-polar solvents.[39] To increase its solubility in water and organic solvents and also to modify its fluorescent properties and binding affinity to HSA, the 9-position of **1** was doubly alkylated to obtain **2**. Starting from 2-nitrofluorene **3**, compound **2** was synthesized in four steps (Scheme 1) in moderate overall yield (see Experimental Section for details). **2** was highly soluble in water as well as in organic solvents. We first compare the absorption and fluorescence spectroscopic characteristics of **1** and **2** in various solvent media, and then describe the binding characteristics of **1** and **2** to HSA. Energy transfer from excited Trp-214 to HSA-bound **2**, and from HSA-bound **2** to Cys-34-bound acrylodan (AC) in the HSA complex are followed. Finally, competitive binding studies of **1** and **2** with other ligands (shown below) of pharmaceutical interest to HSA are presented.



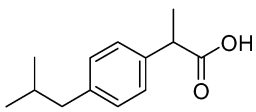
acrylodan (AC)



aspirin



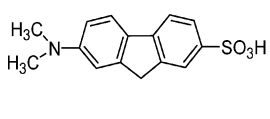
phenylbutazone (PBZ)



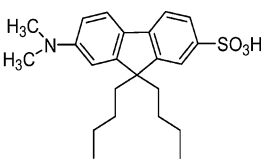
ibuprofen (IBU)

Solvent dependence of UV-visible absorption and fluorescence spectra of 1 and 2

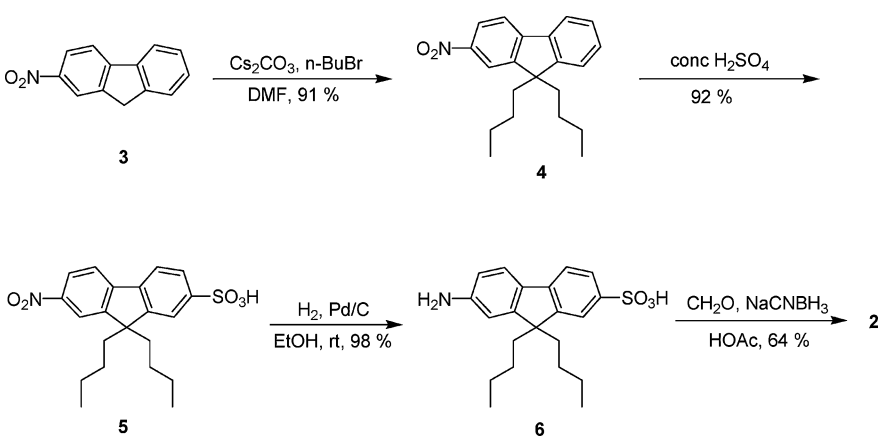
Compounds **1** and **2** show absorption bands in the 260–380 nm region with that of **2** appearing at a slightly longer wavelength than **1**. Though the shape and position of the absorption bands vary somewhat with solvent, no systematic dependence on solvent parameters could be deduced (see ref. 39 for variation of the spectrum of **1** on solvent). Close examination of the spectra reveals that it is a sum of at least two transitions, presumably



1



2



3 4 5 6

Scheme 1 Synthetic pathway to **2**.

Table 1 Comparison of absorption and fluorescence characteristics of **1** and **2**[a]

	Absorption				Fluorescence			
	1		**2**		**1**		**2**	
Medium	λ_{max}/nm	log ε_{max}	λ_{max}/nm	log ε_{max}	λ_{max}/nm	I_{max}[b]	λ_{max}/nm	I_{max}[b]
Water (pH 7.0)	319	4.38	325	4.33	432	1.00	427	1.00
CH_3OH	321	4.39	337	4.38	397	1.11	395	1.16
C_2H_5OH	318	4.40	335	4.36	385	1.12	385	1.23
CH_3CN	314	4.42	318	4.34	383	1.06	383	1.25
2-PrOH	314	4.41	320	4.36	376	1.13	375	1.31
p-dioxane	319[c]	4.44[c]	344	4.38	375	1.15[d]	374	0.99
DMF	—		317	4.39			380	0.90
THF	—		335	4.36			373	1.04

[a] Data for **1** are taken from ref. 39. [b] Relative intensity at emission maxima, normalized to light absorption by dividing the measured intensities by $(1–10^{-Abs})$: Abs is absorbance at λ_{ex}. [c] In 96% *p*-dioxane–4% water. [d] In 98% *p*-dioxane–2% water ($\lambda_{max,em}$ = 378 nm).

to different vibrational states,[39] and the lower energy transition is more preferred in non-polar media. This is more pronounced for **2** than for **1**. The absorption maxima and log ε_{max} values in various solvents are summarized in Table 1.

In a previous report, we showed that the fluorescence sensitivity ($\varepsilon_{max} \times \Phi_F$) of **1** is about 15–25 times greater than that of (dimethylamino)naphthalenes.[39] The fluorescence sensitivity of **2** was similar to that of **1**. In all solvent media studied, the fluorescence spectra of **1** and **2** show a single band which shows blue shift with low solvent polarity. The normalized fluorescence spectra of **2** are shown in Fig. 2 (the spectra of **1** can be found in ref. 39). The emission maxima and relative emission intensities are included in Table 1. The observed dependence of fluorescence spectra on solvent polarity is typical of that of emission from an excited intramolecular charge transfer (ICT) state,[1] and indicates that **1** and **2** can be used as fluorescent probes.

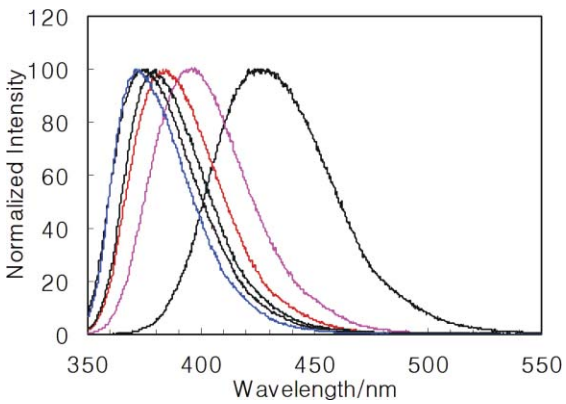


Fig. 2 Normalized fluorescence spectra of **2**. Solvents are H_2O (pH 7), CH_3OH, C_2H_5OH, DMF, 2-PrOH and THF (from right to left of the spectra). [**2**] = 1.9×10^{-6} M. λ_{ex} = 334 nm. (For relative intensity, see Table 1).

Binding of 1 and 2 to HSA

Addition of HSA to the solutions of **1** or **2** (pH 7, phosphate buffer) results in large spectroscopic changes. Fig. 3 shows the absorption spectra of HSA, **2**, and HSA-bound **2**. A red-shift of absorption spectrum of **2** upon binding to HSA, *i.e.* from 325 nm in water to 340 nm in the presence of HSA, is clearly seen. Similarly, the

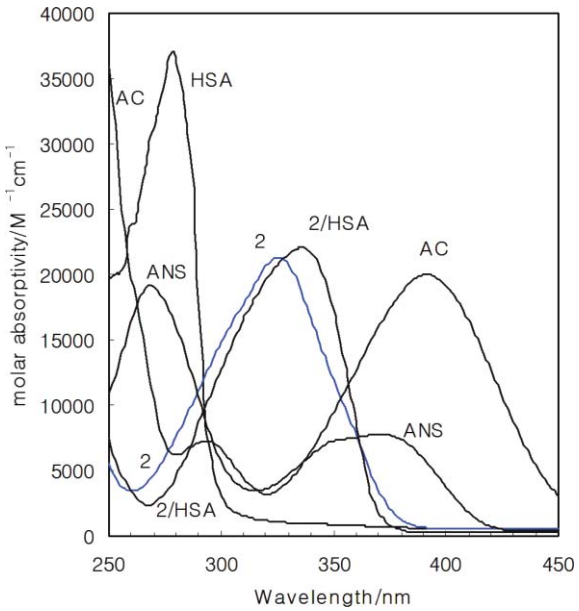


Fig. 3 Absorption spectra of HSA, **2**, HSA-bound **2**, ANS, and acrylodan (AC). The spectrum of HSA-bound **2** is assumed to be that of an equimolar (2.0×10^{-5} M each) mixture of **2** and HSA taken against HSA blank. Solvent media are aqueous 0.01 M phosphate buffer (pH 7.0, I = 0.1 M with NaCl), except ANS and AC which were taken in methanol.

absorption maximum of **1** shifted from 319 nm in water to 335 nm upon addition of HSA (ESI, Fig. S1‡).

The dependence of the fluorescence spectra of **1** and **2** on the concentrations of HSA are shown in Fig. 4. As the concentration of HSA increases, the emission at longer wavelength, which is from free fluorophores, decreases and a new emission band at shorter wavelength, which is from HSA-bound fluorophore, grows. This clearly reflects the binding of **1** and **2** to hydrophobic sites of HSA. At high concentration of HSA, the emission maximum of **1** is 410 nm which is similar to that observed in a 60 (v/v)% methanol–40% water mixture,[39] whereas that of **2** is 377 nm, similar to that in 2-propanol. This suggests that **1** and **2** might bind to hydrophobic sites of HSA of different polarity and/or the exposed extent of the bound fluorophores may be different.

Fluorescence titrations of **1** and **2** with HSA show iso-emissive points only at [HSA]/[F] ⟩⟩ 1 (Fig. 4): F denotes the fluorophore.

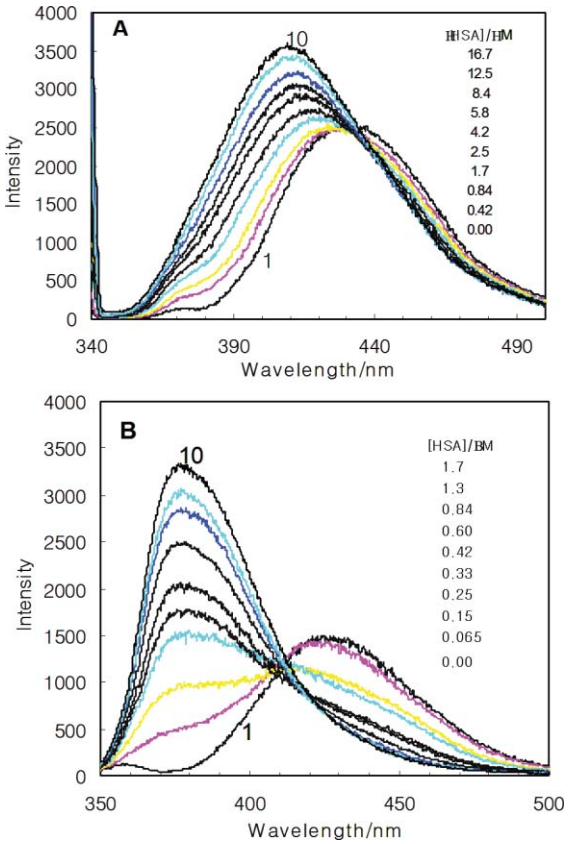


Fig. 4 Fluorescence spectra of 1.0×10^{-7} M **1** (A) and **2** (B) in the presence of various concentrations of HSA. [HSA] are given in the figure. The excitation wavelengths were 330 nm for **1** and 320 nm for **2**.

A plausible explanation for this is that the fluorophores bind to multiple sites of HSA, and the binding is non-cooperative. At a high [HSA]/[F] ratio, there would be little chance of multiple binding, and thus a microscopic binding equilibrium to site *i* can be written as eqn (1) and the fraction (f) of F bound to any site of HSA is given by eqn (2).

$$F + HSA = (F\text{–}HSA)_i;\ K_i = [(F\text{–}HSA)_i]/\{[F][HSA]\} \quad (1)$$

$$f = \Sigma_i\,[(F\text{–}HSA)_i]/[F]_o = K[HSA]/(1 + K[HSA]);\ K = \Sigma_i K_i \quad (2)$$

Eqn (2) is analogous to that derived for 1:1 binding, but the apparent binding constant K equals the sum of the microscopic binding constants. As the observed fluorescence intensity is the sum of the intensities from contributing species, the intensity change (ΔI_F) caused by the presence of HSA is derived as;

$$\Delta I_F/[HSA] = \Delta I_{F,\infty}\,K - \Delta I_F\,K \quad (3)$$

where $\Delta I_{F,\infty}$ is the intensity change expected when all of F is bound to HSA. Under the condition of a large excess of HSA, [HSA] can be replaced by total HSA concentration, $[HSA]_o$.

In Fig. 5, ΔI_F values of **1** and **2** at wavelengths showing the largest emission intensity change as functions of $[HSA]_o$ are plotted. The plots of $\Delta I_F/[HSA]$ *versus* ΔI_F according to eqn (3) are given in the inset of the figure. The plots gave good linearity with the coefficient of correlation >0.98. The K values of 0.37×10^6 M^{-1} for **1** and 2.2×10^6 M^{-1} for **2** were obtained from the plots. The larger binding constant of **2**, compared to **1**, suggests that the dibutyl

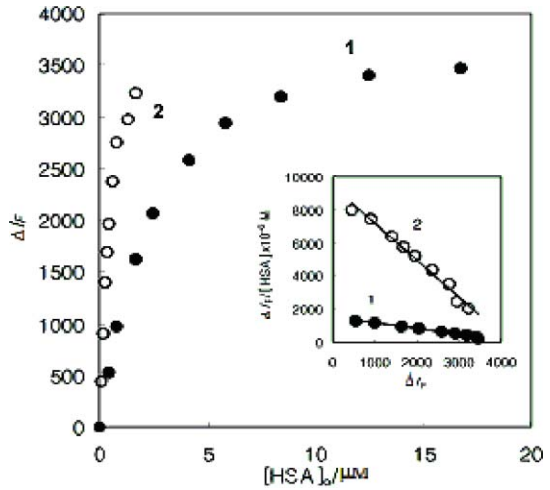


Fig. 5 Changes of fluorescence intensity (ΔI_F) of **1** at 397 nm (●) and **2** at 377 nm (○) with [HSA]. The contributions from HSA to the measured intensities were corrected. The ΔI_F scale was adjusted to show similar $\Delta I_{F,\infty}$ for both systems. Inset shows the plots of $\Delta I_F/[HSA]$ against ΔI_F. [**1**] = [**2**] = 1.0×10^{-7} M.

substituent of **2** increases the binding affinity to HSA. The K value of **2** is significantly larger than those of widely used fluorescent probes for HSA, dansyl derivatives ($0.02–0.5 \times 10^6$ M^{-1}),[9,14,31,32] warfarin (ca. 0.4×10^6 M^{-1}),[31,35] prodan ($0.1–0.4 \times 10^6$ M^{-1}),[27,28] promen (0.2×10^6 M^{-1})[29] and 1,8-ANS (0.9×10^6 M^{-1}).[9,15] From $\Delta I_{F,\infty}$ values, the fluorescence intensities at emission maxima of the HSA-bound **1** and **2** were found to be 1.8 and 3.0 times greater than the respective values in buffer. The enhancement of emission intensities upon binding to HSA are much greater than those in homogeneous media caused by solvent change (Table 1). As we excited the solutions at isosbestic points, the enhancement of emission intensity upon binding to HSA is approximately proportional to the increase in the emission quantum yields of the fluorophores. This may arise from protection of the fluorophores from quenching and/or from decrease in the non-radiative decay. The high binding constant, and large fluorescence quantum yield and molar absorptivity, together with the large fluorescence spectral change upon binding to HSA give promise that **2** could be utilized as a sensitive fluorescent probe even at low concentrations of the probe and HSA.

Resonance energy transfer from Trp-214 to HSA-bound 1 or 2

HSA has a single tryptophan residue (Trp-214) which gives a fluorescence emission in the 300–400 nm region. The emission band of Trp-214 overlaps with absorption bands of **1** and **2**. Thus, Förster resonance energy transfer from the excited Trp-214 to the fluorophores is possible within the complexes. Fig. 6 shows the emission spectra of HSA in the presence of various concentrations of **2**, excited at 282 nm where the tryptophan residue absorbs. At a given concentration of HSA, the tryptophan fluorescence (~340 nm) decreases and the fluorescence from HSA-bound **2** (~380 nm) increases as the concentration of **2** is higher. No appreciable tryptophan fluorescence is observed at high concentration of **2**. Similar results are observed in the presence of **1** (ESI, Fig. S2‡). These indicate near 100% efficiency in the resonance energy transfer from the excited Trp-214 to HSA-bound

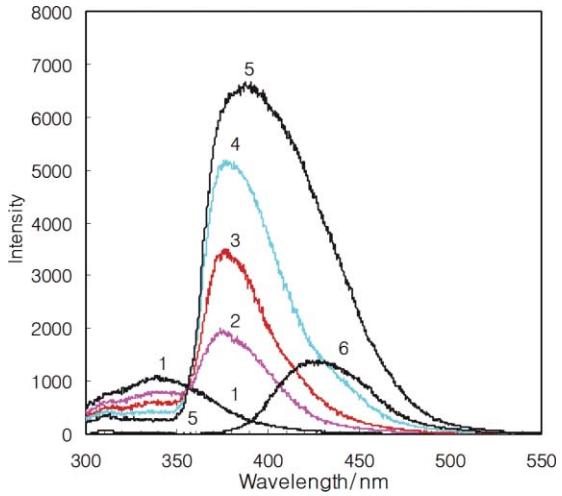


Fig. 6 Fluorescence spectra of HSA/**2** solutions excited at 282 nm. [HSA] = 1.7×10^{-6} M. The concentrations of **2** are (1), 0.00×10^{-6}; (2), 0.47×10^{-6}; (3), 0.94×10^{-6}; (4), 1.9×10^{-6}; (5), 3.8×10^{-6} M. The spectrum 6 is from 0.94×10^{-6} M **2** solution without HSA.

1 and **2**. The efficiency is much greater than the corresponding energy transfer efficiency to prodan[22,28] or promen,[22] which is 38–50%, suggesting that **2** is a better resonance energy acceptor from excited Trp-214.

To estimate the contribution of the Trp-214 sensitized emission to the fluorescence of HSA-bound species, we compared the spectra 3 and 6 of Fig. 6, which were taken with the same concentration of **2** in the presence and in the absence of HSA, respectively. The maximum emission intensity (at 377 nm) of spectrum 3 is 2.4 times greater than that of spectrum 6 (at 427 nm). Unbound **2** contributes little to emission intensity at 377 nm (see Fig. 4B). The fraction of **2** bound to HSA is calculated to be 0.70 from the binding constant of **2** with HSA and the concentrations of **2** and HSA in the solution used for spectrum 3. Thus, it appears that HSA-bound **2** gives 3.4 (= 2.4/0.7) times greater emission intensity than free **2** at their emission maximum when $\lambda_{ex} = 282$ nm. The molar absorptivities of HSA-bound **2** and free **2** are estimated as 5300 M^{-1} cm^{-1} and 8300 M^{-1} cm^{-1}, respectively, at 282 nm (Fig. 3). From these values, it is estimated that the HSA-bound **2** gives 5.3 times greater emission intensity than the free **2** at their respective emission maxima. This is much greater than the ratio 3.0 found in the previous section when the solutions are excited at the isosbestic point of HSA-bound **2** and free **2**. Thus, about 43% of 377 nm emission of HSA-bound **2** can be attributed to the Trp-214 sensitized emission. This estimation matches well with the similarity of molar absorptivities of the HSA-bound **2** and tryptophan (5600 $M^{-1}cm^{-1}$),[2] and the near 100% energy transfer efficiency.

Binding to the HSA–acrylodan conjugate

Acrylodan (AC) was covalently attached to the Cys-34 site of HSA.[23,25,26] Upon binding to HSA, the emission maximum of AC is blue-shifted to *ca.* 480 nm from 525 nm in aqueous buffer, revealing a hydrophobic character of the microenvironment surrounding Cys-34 in HSA. We prepared the HSA–AC conjugate by reacting HSA with AC in a 1:1 molar ratio. No appreciable change in the fluorescence spectrum was observed when the concentration of the reaction mixture was varied from 0.1–10 μM suggesting nearly quantitative covalent bonding of the reacting pair.

Fig. 7 shows three sets of fluorescence spectra (A–C) of a HSA–AC conjugate solution in the presence of various concentrations of **2** taken at different excitation wavelengths. The behavior of the tryptophan fluorescence (Set A: 300–360 nm) taken with λ_{ex} = 290 nm is similar to that observed with free HSA solution in the presence of **2** (Fig. 6). It was reported that 40% of Trp-214 fluorescence of the HSA–AC conjugate is transferred to AC.[22] The remaining Trp-214 fluorescence is further quenched by **2** indicating binding of **2** to the HSA–AC conjugate. The binding is clearly seen in the set B spectra (350–480 nm) taken with $\lambda_{ex} = 320$ nm where mostly **2** absorbs. When the [**2**]/[HSA-AC] ratio is less than 1, the fluorescence spectra of this set are similar to that of AC-free HSA-bound **2**. From analysis of the fluorescence titration data of HSA–AC with **2** using linear regression methods described in a previous report,[42] the binding constant for **2** to the HSA–AC conjugate was estimated to be approximately 1×10^6 M^{-1}. This is similar to the binding constant of **2** to the native HSA. When the solution was excited at 400 nm where only AC absorbs, a slight decrease in AC fluorescence intensity, without noticeable change in spectral shape, was observed (set C). This implies that the bound AC does not alter the environment of the binding site of **2** significantly. Also it appears that the binding of **2** to HSA–AC does not affect the environment of AC.

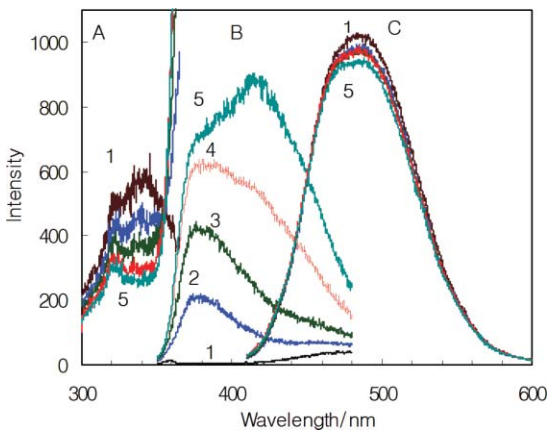


Fig. 7 Fluorescence spectra of **2**/HSA–acrylodan solutions. [HSA–acrylodan] = 1.0×10^{-6} M. The concentrations of **2** are (1), 0×10^{-6}; (2), 0.3×10^{-6}; (3), 0.6×10^{-6}, (4) 1.0×10^{-6}; (5), 1.5×10^{-6} M. The excitation wavelengths were 290 nm for group **A**, 320 nm for group **B**, and 400 nm for group **C**.

Competitive binding with other ligands

To obtain information on the binding site(s) of **1** and **2** in HSA, competitive binding between the fluorene compounds and other ligands such as 1-anilino-8-naphthalene sulfonate (ANS), aspirin, *S*-(+)-ibuprofen (IBU) and phenylbutazone (PBZ) was studied. Because of binding to multiple sites, ambiguity of binding constants,[7-10,14-16,33] and possible allosteric interactions between the ligands bound to different sites,[9,44] it is difficult to analyze the competitive binding data quantitatively. However, qualitative analysis could be carried out using reported binding data for competitors.

The right panel of Fig. 8 shows the effect of **2** on the fluorescence spectra of 5.0×10^{-6} M ANS in 1.2×10^{-6} M HSA solution. As ANS is highly fluorescent in non-polar media or in the hydrophobic pockets of proteins, but very weakly fluorescent in homogeneous aqueous media, the decrease in the ANS fluorescence intensity upon the addition of **2** reflects the displacement of the bound ANS by **2**. It was reported that there are at least two types of fluorescence-active ANS binding sites in HSA.[9,15,16] The high affinity site accommodates one ANS molecule with a binding constant (K) of ca. 0.9×10^{6} M^{-1}, and the low affinity site can bind 2–3 molecules with K values of $0.08–0.13\times10^{6}$ M^{-1}.[9,15] A recent paper reported about 6 times greater binding constants for the respective sites,[16] but these values are too large to account for our results. Competitive binding studies with specific site markers suggested that the primary binding site for ANS is the site II in the subdomain IIIA.[9,15] Bagatolli *et al.* showed that the ANS molecules bound to the low affinity sites contribute about 20% of ANS fluorescence from ANS/HSA solutions under similar conditions employed in this work.[15] The 20% contribution of ANS fluorescence from low affinity sites is much smaller than the observed decrease in the ANS fluorescence intensity upon addition of **2** (43% decrease in the presence of 10×10^{-6} M **2**). This implies that ANS and **2** compete for the site II of HSA, which is the primary binding site of ANS.

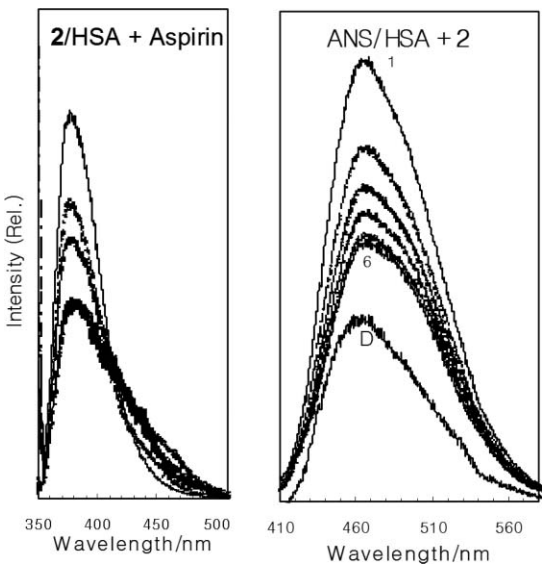


Fig. 8 (Right) Fluorescence spectra of 1,8-ANS/HSA solutions in the presence of various concentrations of **2**. [1,8-ANS] = 5.0×10^{-6} M; [HSA] = 1.2×10^{-6} M. The concentrations of **2** are 0, 1, 2, 3, 5, and 10×10^{-6} M (from top to bottom). The spectrum D is the difference spectrum between the spectra 1 and 6. λ_{ex} = 400 nm. (Left) Effect of aspirin on the fluorescence spectra of **2**/HSA solution. [**2**] = 2.0×10^{-7} M; [HSA] = 1.0×10^{-6} M; [aspirin] = 0, 1.0, 2.0, 3.5, and 5.0×10^{-4} M (from top to bottom). The contributions of aspirin/HSA to the spectra were subtracted from each **2**/HSA/aspirin spectrum. λ_{ex} = 350 nm.

The left panel of Fig. 8 shows the displacement of HSA-bound **2** by aspirin. The addition of aspirin to **2**/HSA solution clearly decreases the emission from HSA-bound **2** (~377 nm) and increases the emission from free **2** (~430 nm). It was shown that aspirin is known to bind to at least two sites located in the subdomains IIA and IIIA of HSA:[8,9,43] the binding constant to the high affinity site was reported to be about 2×10^{4} M^{-1},[9,15,43] whereas that to low affinity site was 1.8×10^{3} M^{-1}.[9,43] Under our experimental conditions of 2.0×10^{-7} M **2** and 1.0×10^{-6} HSA, the fluorescence intensity at 377 nm is decreased to about half by the presence of 5×10^{-4} M aspirin. As the free **2** does not contribute to the fluorescence intensity at 377 nm, the results imply that about half of HSA-bound **2** is displaced by aspirin. If **2** binds to multiple sites on HSA, the extent of displacement from the sites for which it is in competition with aspirin would be greater than this. Ignoring the binding of **2**, the fraction of HSA of which any given site is not occupied by aspirin is given by $1/(1 + K_{aspirin}[aspirin])$. Using the reported $K_{aspirin}$ values given above, the fractions for the high affinity site and low affinity site are calculated as 0.09 and 0.5, respectively, at [aspirin] = 5×10^{-4} M: as aspirin is in large excess of HSA, the total aspirin concentration is used for [aspirin]. If **2** and aspirin compete for the low affinity aspirin binding site, the effect of 5×10^{-4} M aspirin on the fluorescence of 2.0×10^{-7} M **2** is approximately the same as that by decreasing the concentration of HSA to 0.5×10^{-6} M from 1.0×10^{-6} M in the absence of aspirin. As the fraction of **2** bound to HSA (f_{HSA}) is given by $K_{HSA}[HSA]/(1+K_{HSA}[HSA])$, the f_{HSA} value changes to about 0.48 from 0.65 by the addition of 5×10^{-4} M aspirin. This estimated change of f_{HSA} is much smaller than the observed displacement result. Thus we can conclude that **2** is displaced from the high affinity aspirin binding site. As **2** binds mostly to site II, as revealed from displacement of ANS by **2**, we can conclude that the high affinity site for aspirin is also site II. This supports the conclusion from displacement experiments of ANS by aspirin.[15]

We also studied the displacement of HSA-bound **1** by aspirin. A clear displacement was shown, but the extent of the displacement was much smaller than that of **2** (ESI, Fig. S4‡). This implies that the primary binding site for **1** may be different from that of **2**.

More conclusive evidence for the difference in the primary binding site for **1** and **2** was obtained from competitive binding studies using site specific markers, *S*-(+)-ibuprofen (IBU) and phenylbutazone (PBZ). The primary binding site of IBU is reported to be site II,[5,31,33] whereas that of PBZ is site I.[5,8,10,14,31] Fig. 9 shows the relative fluorescence intensities of **1** and **2** in HSA solutions, measured at emission maxima of the respective HSA complexes, as a function of IBU or PBZ concentration. The emission from HSA-bound **1** was increased by the addition of IBU and PBZ. The emission maximum was shifted to shorter wavelength by 2 nm in the presence of 40 μM PBZ. This indicates that **1** is not displaced from HSA by either IBU or PBZ. The enhancement of fluorescence of a probe by the addition of drugs was reported for the case of ANS with iophenoxic acid and furemide,[9] and for dansyl compounds with warfarin[9] and other drugs.[31] This was attributed to sensitivity of the probe to conformational changes in the protein occurring upon binding of the drugs.[31] The blue shift in the emission maximum of **1** caused by PBZ suggests that the PBZ-induced conformational change makes the environment of bound **1** more hydrophobic.

The addition of IBU decreased the fluorescence from HSA-bound **2** and increased the emission from free **2**, whereas PBZ had little effect on **2**. This confirms that the primary binding site of **2** is site II, which is consistent with the conclusion obtained from displacement studies of ANS by **2**, and **2** by aspirin (*vide supra*).

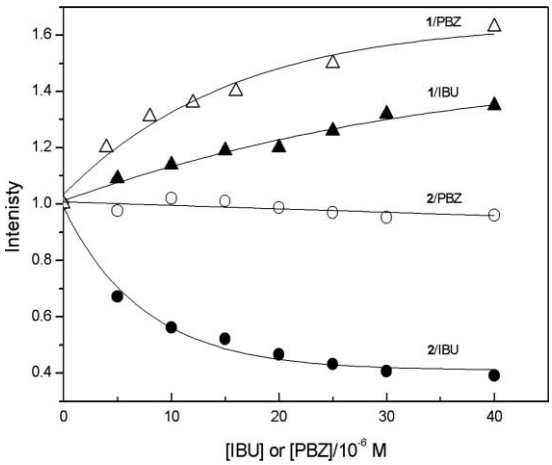


Fig. 9 Effect of *S*-(+)-ibuprofen (●, ▲) and phenylbutazone (○, △) on the **1**/HSA (▲, △) and **2**/HSA (●, ○) solutions. **1**/HSA: [**1**] = 5.0×10^{-7} M, [HSA] = 5.0×10^{-6} M; **2**/HSA: [**2**] = 5.0×10^{-7} M, [HSA] = 1.0×10^{-6} M. The fluorescence intensities are measured at emission maxima of the HSA-bound fluorophores, 397 nm for **1** and 376 nm for **2**.

Conclusive information on the binding site of compound **1** could be given by X-ray crystallographic analysis, but this is beyond the scope of this work. It is clear that **1** does not bind to site II, which is smaller than site I:[6] otherwise it would be displaced by IBU which has the same negative charge. One possibility is that **1** binds to a site other than sites I and II. A third high affinity site located in domains other than IIA and IIIA was reported for fatty acids and other drugs.[6,9,10] Another possibility is that **1** binds to site I. This is supported by the weak displacement of **1** by aspirin. The cavity of site I is large and has three regions for ligand binding.[6,14] Due to their relatively small sizes, **1** and PBZ may cooperatively bind to site I. The effect of IBU on the binding of **1** can be attributed to cooperative allosteric interaction of the ligands. Such interaction between ligands bound to sites I and II has been reported for other ligand pairs.[44]

Conclusions

A novel water-soluble fluorescent molecule, 9,9-dibutyl-7-(dimethylamino)-2-fluorenesulfonate **2** has been successfully prepared from 2-nitrofluorene in moderate overall yield. The fluorescence spectrum of **2**, as well as that of 7-(dimethylamino)-2-fluorenesulfonate **1**, is blue-shifted with decreasing polarity of medium or with binding to human serum albumin (HSA). The apparent binding constants to HSA are 0.37×10^6 M^{-1} for **1** and 2.2×10^6 M^{-1} for **2**. The primary binding site on HSA is site II in domain IIIA for **2**, whereas that of **1** is believed to be site I in domain IIA, but involving a different region from the phenylbutazone binding site. The aminofluorene-based fluorescent molecules, particularly **2**, exhibit many desirable characteristics for sensitive fluorescent probes, such as high molar absorptivity, strong fluorescence, and a high sensitivity of the fluorescence to environmental changes. This, together with the large binding constant and site specificity to HSA will make them useful as fluorescent probes for proteins and other biological molecules, potentially at one-order of magnitude lower concentrations than the widely used aminonaphthalene-based probes.

Experimental

Synthesis of 9,9-dibutyl-7-(dimethylamino)-2-fluorenesulfonic acid 2

To a stirred reaction mixture of **3** (1.055 g, 5 mmol) and caesium carbonate (4.885 g, 15 mmol) in DMF (25 mL) was added *n*-butyl bromide (2.054 g, 1.62 mL, 15 mmol) under a nitrogen atmosphere and stirring was continued at room temperature for 16 h. Water (30 mL) was added to the reaction mixture which was then extracted with dichloromethane (3 × 30 mL). The organic layers were dried, concentrated, and then purified by column chromatography (eluent: dichloromethane: hexane = 10:3) to give **4** as light yellow solid (1.48 g, 91%). **4**: Mp, 72–73 °C; ^{1}H NMR (CD_2Cl_2, 500 MHz): δ 8.23 (dd, J = 8.5, 2.0 Hz, 1H), 8.20 (d, J = 2.0 Hz, 1H), 7.82-7.79 (m, 2H), 7.44-7.37 (m, 3H), 2.09-1.99 (m, 4H), 1.06 (sextet, J = 7.4 Hz, 4H), 0.64 (t, 6H, J = 7.4 Hz), 0.57-0.49 (m, 4H); ^{13}NMR (CD_2Cl_2, 125 MHz): δ 152.46, 152.08, 147.79, 147.34, 138.94, 129.30, 127.43, 123.39, 123.21, 121.23, 119.88, 118.39, 55.74, 39.89, 26.04, 23.01, 13.62.

A reaction mixture of **4** (1.30 g, 4.02 mmol) and concentrated sulfuric acid (8.0 mL) was stirred at room temperature for 24 h and then poured into cold water (30 mL) to give precipitates, which were filtered, washed with water and dried. More products were recovered from the filtrate by extraction with ethyl acetate (3 × 25 mL). The residue from ethyl acetate layers and the precipitates were combined and purified by column chromatography (eluent: ethyl acetate and then ethyl acetate:methanol = 8:1) to give **5** as light yellow solid (1.50 g, 92%). **5**: Mp > 200 °C (dec); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.36 (d, J = 2.0 Hz, 1H), 8.27 (dd, J = 8.0, 2.0 Hz, 1H), 8.10 (d, J = 8.0 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.74 (s, 1H), 7.71 (d, J = 8.0 Hz, 1H), 2.20–2.00 (m, 4H), 1.05 (sextet, J = 7.4 Hz, 4H), 0.63 (t, J = 7.4 Hz, 6H), 0.51–0.44 (m, 4H).

Compound **5** (0.66 g, 1.64 mmol) was reacted with 1 atmospheric hydrogen gas in the presence of 5% Pd/C (0.15 g) at room temperature for 5 h. Filtration of the reaction mixture followed by concentration of the filtrate afforded **6** (0.60 g, 98%). **6**: Mp > 250 °C (dec); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.51–7.41 (m, 4H), 6.58 (d, J = 2.0 Hz, 1H), 6.53 (dd, J = 8.0, 2.0 Hz, 1H), 5.26 (broad s, 2H), 1.93–1.75 (m, 4H), 1.06 (sextet, J = 7.4 Hz, 4H), 0.65 (t, J = 7.4 Hz, 6H), 0.57–0.45 (m, 4H).

To a mixture of **6** (0.366 g, 0.975 mmol) and paraformaldehyde (0.360 g, 12.0 mmol) in glacial acetic acid (10 mL) was added sodium cyanoborohydride (0.315 g, 5.0 mmol). After stirring at room temperature under a nitrogen atmosphere for 18 h, the reaction mixture was poured into ice water (10 mL) to obtain precipitates. The precipitates were filtered, washed with water, and put in hot water (10 mL) and then basified with 30% ammonia solution to obtain a clear solution. The solution was acidified to ca. pH 3 with 1 N HCl to produce precipitates, which were filtered, washed with water and then vacuum-dried to give the product **2** (97 mg). More products were recovered from the filtrate by extraction with ethyl acetate (5 × 15 mL). The residue from the ethyl acetate layers was purified by reverse-phase medium pressure liquid chromatography (flow rate 20 mL/min, CH_3CN–1% TFA/H_2O–1% TFA gradient) to give the product (0.152 g). The combined yield of **2** from **6** was 0.249 g (64%). Mp, 164–166 °C; ^{1}H NMR (CD_3OD, 500 MHz, residual solvent peak

δ = 4.840, 3.309): δ 7.96 (d, J = 8.0 Hz, 1H), 7.91 (s, 1H), 7.89–7.83 (m, 2H), 7.71 (s, 1H), 7.58 (d, J = 7.5 Hz, 1H), 3.36 (s, 6H), 2.17–2.05 (m, 4H), 1.07 (sextet, J = 7.4 Hz, 4H), 0.65 (t, J = 7.4 Hz, 6H), 0.62–0.45 (m, 4H); ^{13}C NMR (CD_3OD, 125 MHz, residual solvent peak δ = 49.05): δ 155.39, 152.38, 146.44, 143.68, 143.62, 142.48, 126.70, 123.17, 121.93, 121.51, 120.91, 116.64, 57.37, 47.72, 40.78, 27.24, 23.94, 14.19. Anal. Calcd for $C_{23}H_{31}NO_3S{\cdot}H_2O$: C, 65.84; H, 7.93; N, 3.34; S, 7.64. Found: C, 65.60; H, 7.76; N, 3.66; S, 7.54%.

The ammonium salt of **2** was obtained by adding 30% ammonia solution to an aqueous suspension of **2**, followed by evaporation of the solvent.[39]

Other materials and methods

HSA (essentially fatty acid free) and acrylodan (AC) were obtained from Sigma and Molecular Probes, respectively. Other chemicals were of reagent grade quality, obtained from commercial sources and were used without further purification. The solvents used for the spectroscopic measurements were spectroscopic grade. The aqueous solutions were prepared with a phosphate buffer (0.01 M phosphate, pH 7.0, I = 0.1 M with NaCl). HSA was dissolved in the buffer and the concentration was calculated from absorbance data using ε_{280} = 36,600 M^{-1} cm^{-1}.[22,26] The concentrations of others were based on the weights. HSA/AC conjugate was prepared by using Kamal's method with minor modifications:[26] HSA and AC (5×10^{-6} M each) in phosphate buffer were reacted for 24 hrs at room temperature. Stock solutions of **1**, **2**, AC, ANS, aspirin, ibuprofen and phenylbutazone were prepared in methanol. To make a desired solution for spectral measurement, a predetermined amount of a stock solution was taken, methanol was evaporated off, and then an appropriate solvent, buffer or HSA solution was added.

Absorption spectra were taken with an Agilent 8453 spectrophotometer. Fluorescence spectra were obtained with a Hitachi F4500 spectrofluorimeter. The solutions containing HSA were equilibrated for at least 10 hrs, prior to spectral measurements. All spectra were taken at room temperature, 22 ± 2 °C.

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