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Fluorescence properties of albumin blue 633 and 670 in plasma and whole blood

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Abstract. We have determined the fluorescence characteristics of two long wavelength dyes, albumin blue 633 (AB633) and 670 (AB670), in plasma and blood to evaluate the possibility of making direct fluorescence sensing measurements in blood. Using binding and lifetime measurements we were also able to show that these dyes bind selectively to human serum albumin (HSA) in plasma and blood. By measuring changes in the mean lifetime of AB670 with changes in the HSA concentration, we showed that lifetime-based sensing can be used to monitor HSA concentrations using these albumin blue dyes. Anisotropy measurements for AB633 and AB670 in plasma and blood revealed high anisotropy values for these dyes in these media. Exploiting these high anisotropies, we were also able to determine HSA concentrations in plasma and blood mimics using changes in AB670 anisotropy with HSA concentration. These results show that, apart from being able to make fluorescence measurements directly in plasma and blood, it is possible to sense directly for specific plasma/blood components using fluorescent probes that bind preferentially to them.
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1 Introduction

Recently, we showed that fluorescence measurements can be made directly in blood using a one photon excitation of a near infrared (NIR) emitting dye, rhodamine 800.[1] This development we believe could open up the possibilities of making routine clinical measurements directly in blood using fluorescence methods. Such measurements will require fewer preparation steps which will translate to fewer sources of error and faster analysis times. This will also minimize health hazards associated with extensive handling and processing of blood samples.

In this report, we further demonstrate the ability to make fluorescence determinations directly in blood using two red emitting dyes, albumin blue 633 (AB633) and albumin blue 670 (AB670), that have been shown to be specific for human serum albumin (HSA) in urine samples.[2] Here, we show that these dyes, though not completely specific, are very selective for HSA in plasma and blood and can be used to monitor HSA levels in these media. The observed selectivity of these dyes in blood promises the possibility of identifying and designing other red and NIR dyes that could be used to selectively monitor other blood moieties as had been previously suggested.[1]

AB633 and AB670 have been selected for this study since they emit in the red spectral region away from the absorption bands of hemoglobin. Beneficially, these dyes can be excited by red light emitting diodes or red laser diodes which makes them a practical choice for clinical intensity and lifetime-based sensing.

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2 Materials and Methods

AB633 and AB670 were kindly provided by Dr. Otto Wolfbeis. Stock solutions of these dyes were prepared in isopropanol and stored in the dark until use in desired media. It has been previously shown that in aqueous solutions, AB633 and AB670 are quite unstable, having half lifetimes of 72 and 3 h, respectively. However, in isopropanol solutions, they become substantially more stable with AB633 being stable for about 1 yr and AB670 for at least four weeks.[2] Globulin free HSA, gamma globulin, low density lipoprotein (LDL) and high density lipoprotein (HDL) were obtained from Sigma (St. Louis, MO). Phosphate buffered saline (PBS) *p*H 7.40 was obtained from NIH media unit (Bethesda, MD).

Whole blood was obtained from one of the authors (O. O. A.). For experiments in blood, whole blood was used as obtained, at a hematocrit (Hct) of about 40.5% (Hb = 13.3 g/dl). Plasma was obtained from the whole blood by centrifuging at 5000 g using a Beckmann Avanti J-25 I centrifuge. At the end of the centrifugation, the plasma (supernatant) was carefully aspirated off and used for experiments as desired.

All fluorescence measurement were done with front face illumination[1,3–4] in order to minimize scatter and/or light absorption in plasma and blood. Steady state intensity and anisotropy measurements were performed with an SLM 8000 spectrofluorometer (SLM Instruments, Urbana Champaign, IL) using a xenon arc lamp excitation light source.

Steady state concentration dependent anisotropy measurements were made with AB670 (3 μM) in 30–2000 mg/L

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HSA, in PBS and in 10% Hct washed red blood cell (RBC) suspensions. Excitation was at 650 nm and observation at 678 nm. AB670 was used as the representative dye to test the concentration dependent anisotropy profile of the albumin blue dye binding to HSA in plasma and blood.

Frequency domain intensity decay measurements were performed as described previously.[5–8] Excitation at 600 nm was provided by the fundamental output of a rhodamine 6G dye laser which was synchronously pumped by a mode-locked argon ion laser. The dye laser was cavity dumped at 3.77 MHz. Phase angle and modulation measurements at frequencies higher than 3.77 MHz were performed using the harmonic content of the picosecond pulses.[9,10] Phase angles and modulations were measured relative to scattered light at 600 nm using a 600 nm interference filter in some instances and relative to rhodamine 800 ($\tau = 0.68$ ns) in other instances. Emission was observed at 630 nm using a 630 nm interference filter for AB633, and at 670 nm using a 670 nm interference filter for AB670.

Time domain measurements were performed as previously described.[11–13] Excitation at 600 nm was done by the fundamental output of a rhodamine 6G dye laser synchronously pumped by a mode-locked argon ion laser and cavity dumped at 3.77 MHz. Emission was also observed at 630 nm for AB633 and 670 nm for AB670 using the appropriate interference filters. The instrument response factor was determined from the time profile of the scattering obtained from a diluted ludox solution. A microchannel plate-photomultiplier tube detector was used for detection in these measurements. All fluorescence measurements in 4 g/dl HSA, plasma, and whole blood were performed using front face illumination and detection under magic angle conditions.

The time resolved intensity decay for AB633 and AB670 in the different media was determined from both the frequency domain and time domain data using the multiexponential model

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i), \tag{1}$$

where α_i and τ_i are the pre-exponential factors and decay times, respectively. The fractional contribution of each decay time to the steady state intensity was given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \tag{2}$$

while the mean lifetime was given by

$$\tau = \sum_i f_i \tau_i = \frac{\sum_i \alpha_i \tau_i^2}{\sum_j \alpha_j \tau_j}. \tag{3}$$

3 Binding Measurements

In order to determine fractional fluorescence intensities of AB633 and AB670 associated with dye bound to different macromolecular components in plasma and whole blood, we need to measure and analytically describe binding isotherms of the dyes to the major blood components including HSA, γ globulin, LDL, HDL, and red blood cells. Since the detailed mechanism of binding is not known and might differ for different macromolecules, we have used as an approximation a simple phenomenological approach which will allow us to make rough estimates of the binding of the dyes in the mixture of several components.

We assumed that the interaction of these dyes, D, with the different macromolecules, P_k, can be described by the scheme

$$P_k + D \overset{K_{d,k}^{\mathrm{App}}}{\longleftarrow\!\!-\!\!-\!\!\longrightarrow} P_k D. \tag{4}$$

Then the fraction of the dye bound to the macromolecules can be calculated by

$$f_{b,k} = \frac{[P_k D]}{[D] + [P_k D]} = \frac{[P_k]/K_{d,k}^{\mathrm{App}}}{1 + [P_k]/K_{d,k}^{\mathrm{App}}}, \tag{5}$$

where $K_{d,k}^{\mathrm{App}}$ are apparent dissociation constants for the different macromolecules. To account for the observed sigmoidal shape of the binding curves, we have semiempirically introduced a coefficient β_k into Eq. (5):

$$f_{b,k} = \frac{[P_k D]}{[D] + [P_k D]} = \frac{([P_k]/K_{d,k}^{\mathrm{App}})^{\beta_k}}{1 + ([P_k]/K_{d,k}^{\mathrm{App}})^{\beta_k}}. \tag{6}$$

Since $[D] + [P_k D] = [D]_0$, is the total dye concentration, and $[P_k] + [P_k D] = [P_k]_0$, the total macromolecule concentration, Eq. (6) can be rewritten as

$$[P_k]_0 - [P_k] = [D]_0 \frac{([P_k]/K_{d,k}^{\mathrm{App}})^{\beta_k}}{1 + ([P_k]/K_{d,k}^{\mathrm{App}})^{\beta_k}}. \tag{7}$$

Equation (7) can be numerically solved for any experimental combinations of $[D]_0$ and $[P_k]_0$, thereby giving us the free macromolecule concentration, $[P_k]$, as a function of the parameters $K_{d,k}^{\mathrm{App}}$ and β_k. From this, the fraction of the bound dye, $f_{b,k}$ can then be calculated from Eq. (5).

The fluorescence intensity, I_k, of such an interacting system can be given by

$$I_k = f_{b,k} I_{b,k} + (1 - f_{b,k}) I_{f,k}, \tag{8}$$

where I_b and I_f are intensities of the bound and free dyes, respectively. Fitting of Eq. (8) to the experimental titration curves gives us the values of $K_{d,k}^{\mathrm{App}}$ and β_k.

In a system like ours, where dyes can bind to multiple components, the bulk dye concentration is described by

$$[D]_0 = [D] + \sum_k [P_k D]. \tag{9}$$

With the values of $K_{d,k}^{\mathrm{App}}$ and β_k known, and substituting $[P_k] = [P_{0,k}] - [P_k D]$ in Eq. (7), the fractional intensities, $F_k = I_k / \Sigma I_k$, for the different macromolecules in our multicomponent system can then be calculated by simultaneously solving Eqs. (7), (8), and (9) for all the different macromolecules.

Using this model, we have been able to fit our experimental data adequately.

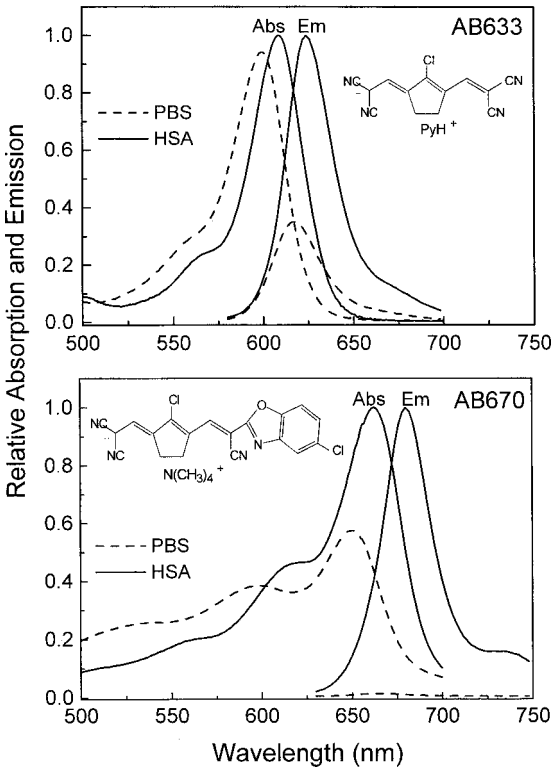

Fig. 1 Relative absorption and emission spectra of 3 μM AB633 (top) and 3 μM AB670 (bottom) in PBS (--) and 100 mg/L HSA (—). Insets are the respective structures of the dyes.

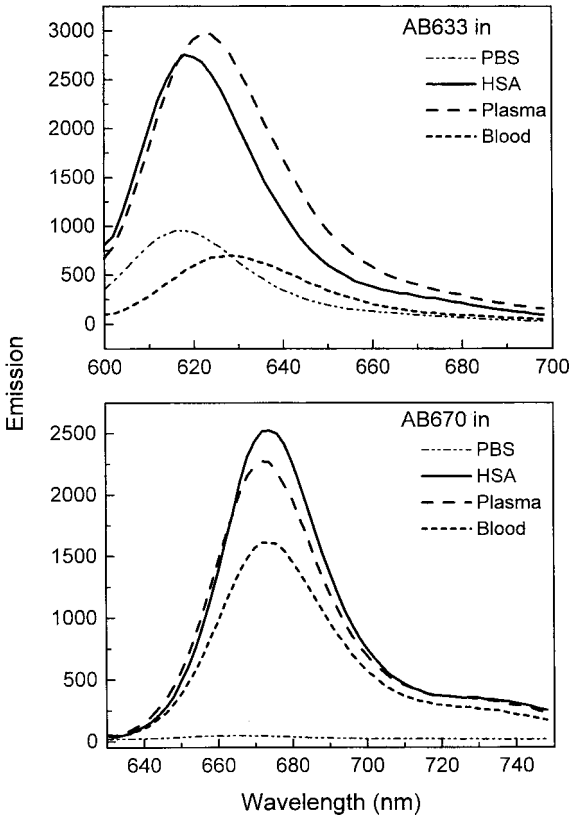

Fig. 2 Relative emission spectra of 3 μM AB633 (top) and 3 μM AB670 (bottom) in PBS (-·--), 4 g/dl HSA (—), plasma (--), and whole blood (-·-). Front face geometry was used for measurements in HSA, plasma and blood.

4 Results

4.1 General Spectral Properties

In Figure 1 we see the absorption and emission spectra of AB633 and AB670 in PBS and HSA. While these dyes absorb and emit above 600 nm in PBS, a shift to the red is further observed in their absorption and emission spectra in HSA. These properties make it possible for these dyes to be excited above 600 nm where autofluorescence, and absorption by hemoglobin (Hb) in red cells is minimal. In addition to the shifts, we also observe an enhancement in absorption and emission in HSA, with this enhancement being more pronounced for AB670 (Figure 1, bottom). In fact we see only very little emission in buffer for AB670 suggesting very little fluorescence contribution from AB670 in its free form in aqueous environments. This characteristic of AB670 we believe would help minimize interference from free dye emission when measurements are made in plasma or blood.

Figure 2 shows the relative emission spectra of 3 μM AB633 (top) and AB670 (bottom) in PBS, 4.0 g/dl HSA (mean blood content), plasma, and whole blood. In general we observe a shift to the red in the emission of AB633 and AB670 in HSA, plasma, and blood indicating interaction of these dyes with these media components. We also observe quenching of the fluorescence of both dyes in whole blood relative to the plasma samples. This quenching we attribute to absorption by Hb in the red cells, an observation that had been previously made with Rh800 in blood.[1] This quenching we also observe to be more pronounced for AB633 in comparison to AB670. This observation we attribute to the greater proximity of the emission band of AB633 to the absorption bands of Hb.

In addition to our intensity measurements, we also determined steady state emission anisotropies for these dyes in HSA (4.0 g/dl), plasma, and whole blood. For both dyes, we obtained high and similar emission anisotropy values of about 0.36–0.37 in both HSA and plasma. The high anisotropy values suggest that the dyes are bound to high molecular weight macromolecules. In whole blood we observe reduced anisotropy values, about 0.23–0.29, for both dyes. Such reduced anisotropy we had observed previously with Rh800[1] in blood, and we had attributed them to multiple scattering events due to the presence of RBC membranes in whole blood. In spite of the drop in the anisotropy values in blood, the obtained values are high enough to be useful for anisotropy/polarization sensing in blood using both dyes.

4.2 Binding Measurements

The ability to identify red/NIR emitting dyes that would preferentially bind to specific components in the blood would be a useful development in clinical chemistry since they can be used to monitor different blood components. Here, we demonstrate the selective binding of AB633 and AB670 to HSA in plasma and blood, in the presence of large gamuts of other

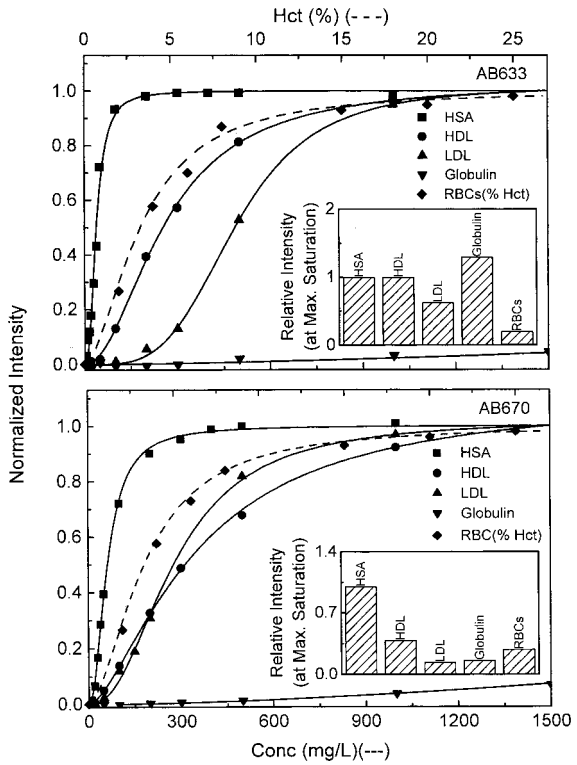

Fig. 3 Binding curves for AB633 (top) and AB670 (bottom) in HSA (■), HDL (●), LDL (▲), globulin (▼), and in washed red blood cells (-◆-). Inset shows the relative emission of the different blood components when all the dye is bound.

macromolecules (proteins, lipids, lipoproteins, etc.) not usually present in the urine where these dyes had been previously found to be specific for HSA.

To establish this characteristic of these dyes in HSA, we measured binding isotherms and relative emission at saturation for the major components in plasma/blood including HSA, γ globulin, LDL, HDL, and RBCs in order to estimate the fraction of total dye bound to each component and the fractional fluorescence intensity associated with the component. RBCs were included in these measurements since in addition to their lipid bilayer, which had been shown to bind weakly to these dyes, they also contain protein moieties which could act as binding sites. Steady state anisotropy and lifetime measurements in di-oleic phosphatidylcholine had earlier suggested very weak binding of these dyes to lipids (results not shown). For parametrization of the measured curves we used Eqs. (4)–(9). The fits adequately represent the experimental data and provide estimates of apparent dissociation constants. Although the apparent $K_{d,k}^{\mathrm{App}}$ can be estimated directly from the raw data without any model assumptions, the analytical representation of the data is needed for simulating the dye behavior in a mixture. Figure 3 shows the normalized binding curves for AB633 (top) and AB670 (bottom) for the different blood components. The insets show the relative intensities for the bound forms of the dyes in the presence of the different components. In Tables 1 and 2, we see the binding parameters for AB633 and AB670, respectively. We observe that in addition to being the most common plasma component (4.1) g/dl, and over 50% of plasma components, HSA also has a very high affinity for AB633 ($K_{d,k}^{\mathrm{App}} = 39$ mg/L or 0.59 μM (Table 1) and AB670 ($K_{d,k}^{\mathrm{App}} = 62$ mg/L or 0.94μM) (Table 2). We noticed that the binding to the other components is relatively weak, with γ globulin having extremely low affinities. In addition, we observed that the reaction between HSA and the dyes is much faster compared to the other components (results not shown). Simulations show that AB633 and AB670 preferentially bind to HSA in a mixture of the components with concentrations adjusted to levels found in plasma or blood, Tables 1 and 2. Our simulations suggest that in an equilibrium the fraction of dye bound to HSA is close to unity, making fluorescence intensity contributions from the other components negligible. It is difficult to test all blood/plasma components, but based on our results, we expect AB633 and AB670

Table 1 Binding parameters for AB633 in different blood components.

Blood component	Mean blood content (mg/L)	$K_{d,k}^{\mathrm{App}}$ (mg/L)	Coefficient β [f]	Q_r [b]	f_b [c,e]	F_i [d,e]
HSA	41 000	39	2.5	2.4	1.000	1.000
HDL	850	260	2.0	2.4	2.1×10^{-7}	2.1×10^{-7}
LDL	810	490	3.8	1.5	1.8×10^{-7}	1.1×10^{-7}
Globulin	32 000	12 000	1.5	3.1	2.2×10^{-7}	1.6×10^{-7}
Red blood cells[a]	43.5[a]	3.0[a]	1.8	0.5	2.4×10^{-6}	5.0×10^{-7}
Free dye	⋯	⋯	⋯	1.0	2.2×10^{-6}	9.0×10^{-9}

[a] Units in % hematocrit.

[b] Relative intensity increase for dye after complete binding to blood component.

[c] Fraction of the total dye bound to the given component in blood.

[d] Calculated fractional intensity of the component in the whole blood, $F_k = I_k / \Sigma I_k$.

[e] Dye concentration, $[D_0]$, is 3 μM for both AB633 and AB670.

[f] See Eq. (6).

Table 2 Binding parameters for AB670 in different blood components.

Blood component	Mean blood content (mg/L)	$K_{d,k}^{App}$ (mg/L)	Coefficient β	Q_r	f_b	F_i
HSA	41 000	62	2.1	330	1.000	1.000
HDL	850	350	1.5	130	3.6×10^{-6}	1.4×10^{-6}
LDL	810	280	2.3	47	1.3×10^{-5}	1.8×10^{-6}
Globulin	32 000	6500	1.7	53	1.5×10^{-5}	2.4×10^{-6}
Red blood cells	43.5	2.9	1.8	97	1.2×10^{-4}	3.5×10^{-5}
Free dye	⋯	⋯	⋯	1	1.0×10^{-6}	3.0×10^{-9}

to bind preferentially to HSA in both plasma and blood, since the macromolecules used in this study constitute the bulk of the components in these media.

4.3 Lifetime Measurements

Figure 4 shows the frequency response curve for AB633 (solid lines) and AB670 (dashed lines) in PBS and HSA. In PBS both dyes exhibit very short lifetimes of about 90–100 ps. However, in 200 mg/L HSA, their mean lifetimes increase dramatically to about 1.02 ns for AB633 and 2.25 ns for AB670, reflecting the enhanced fluorescence observed upon binding in these media. These data were fitted to a two decay time model except for AB633 in PBS that required a one decay time model.

Table 3 shows the lifetime parameters obtained for AB633 in PBS, 4 g/dl HSA, plasma, and whole blood using time domain fluorimetry. Front face illumination and detection was used for these measurements. Acquired data for HSA, plasma, and blood were adequately fit to a three decay time model. The data for PBS was, however, fit to a one decay time model as was done with the frequency domain data (Figure 4). Similar individual lifetimes of 3, 1, and 0.3 ns were obtained for AB633 in HSA and plasma, further supporting the selective binding of AB633 to HSA in plasma. The different lifetimes also suggest heterogenous binding environments or multiple sites on the HSA molecule. The absence of the 100 ps component in the measurements also suggests complete binding of the dye to HSA in both HSA and plasma. In whole blood, the values of the two longer-lived components were found to decrease from 3 and 1 ns to 2.7 and 0.6 ns, respectively. This decrease we attribute to the quenching caused by Hb in the red cells. The mean lifetime was, however, similar to that obtained in plasma.

Table 4 shows the lifetime parameters obtained for AB670 in PBS, 4 g/dl HSA, plasma, and whole blood also using time domain fluorimetry. All the data were fitted to a two decay time model. Compared to the individual lifetimes in PBS, we observed a large increase in the individual lifetimes in HSA, plasma, and whole blood reflecting the enhanced emission observed in these media with AB670. Furthermore, the absence of the 100 ps component also indicates the complete binding of AB670 in these media. The individual lifetimes obtained for HSA, plasma, and blood are also similar, further supporting the selective binding of AB670 to HSA in plasma

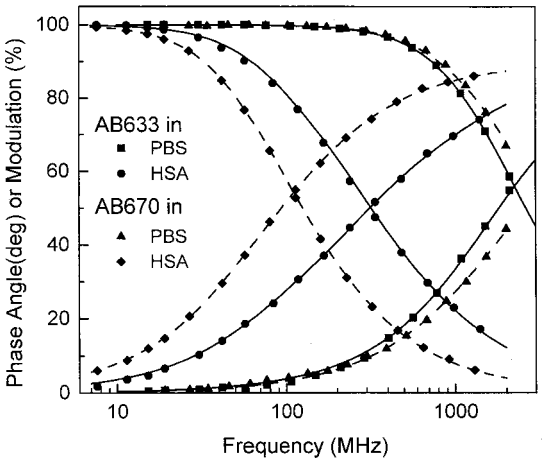

Fig. 4 Phase angles and modulations of AB633 in PBS (■) and HSA (●), and AB670 also in PBS (▲) and HSA (◆). Excitation was at 600 nm, while observation was at 630 nm for AB633 and 670 nm for AB670. Dye concentrations was 3 μM while HSA concentration was 200 mg/L.

Table 3 Lifetimes of AB633 in different media using time domain fluorimetry.

Media	τ_i (ns)	α_i	f_i	$\bar{\tau}$ (ns)	χ_R^2
PBS	0.10	1.00	1.00	0.10	1.24
HSA (4 g/dl)	2.95	0.07	0.28	1.36	1.42
	1.02	0.29	0.44		
	0.30	0.64	0.28		
Plasma	3.02	0.07	0.32	1.46	1.68
	1.12	0.23	0.38		
	0.30	0.70	0.30		
Blood	2.71	0.12	0.47	1.53	1.56
	0.60	0.33	0.33		
	0.30	0.55	0.20		

Table 4 Lifetimes of AB670 in different media using time domain fluorimetry.

Media	τ_i (ns)	α_i	f_i	$\bar{\tau}$ (ns)	χ_R^2
PBS	0.15	0.19	0.36	0.09	1.46
	0.06	0.81	0.64		
HSA (4 g/dl)	2.58	0.73	0.93	2.44	1.29
	0.52	0.27	0.07		
Plasma	2.37	0.70	0.93	2.23	1.34
	0.42	0.30	0.07		
Blood	2.34	0.61	0.84	2.09	1.49
	0.69	0.39	0.16		

and blood. The very slight drop in the mean lifetime of the dye in blood we believe is due to quenching by Hb in blood.

In general, lifetime measurements of AB633 and AB670 in HSA, plasma, and blood further support the selective binding of these dyes to HSA in plasma and blood. In addition, in comparison to the significantly lower intensities observed in whole blood with these dyes, the lifetime values observed with these dyes in blood are similar to those in plasma. This suggests that lifetime measurements are less sensitive to quenching by hemoglobin in red cells. This characteristic we believe could be an advantage in the development of possible lifetime-based sensing techniques for HSA using these dyes as sensors.

5 Lifetime-Based Sensing of HSA Using AB670

In Figure 5, we see the frequency response curve for AB670 at different HSA concentrations. We observe a shift to the left in the frequency response curve as the concentration of HSA increases, indicating an increase in mean lifetime with increasing HSA concentration. The data were globally analyzed in terms of three lifetimes, two lifetimes (2.55 and 1.48 ns) representing the bound dye and the third lifetime (0.1 ns) the free dye. These lifetimes adequately fitted all the HSA concentrations. Figure 6 shows the variation of the mean lifetime (top) and the fractional intensities (bottom) of AB670 with different HSA concentrations. We observe a near linear increase in concentration from about 30 mg/L (0.3 μM) to 200 mg/L (3.0 μM), followed by a gradual leveling off (Figure 6, top). The inset shows the expanded HSA scale in the low concentration region. These results show that it is possible to use lifetime-based sensing to determine HSA concentrations using AB670. Figure 6 (bottom) shows the variation of the calculated fractional intensities for the observed lifetimes. For the free dye (represented by the shortest lifetime, $\tau = 0.10$ ns) we observe a drop in fractional intensity followed by a leveling off at about 150 mg/L. This also suggests that at about 150 mg/L, all the free dye available in the system would have been bound to HSA under our conditions of measurement.

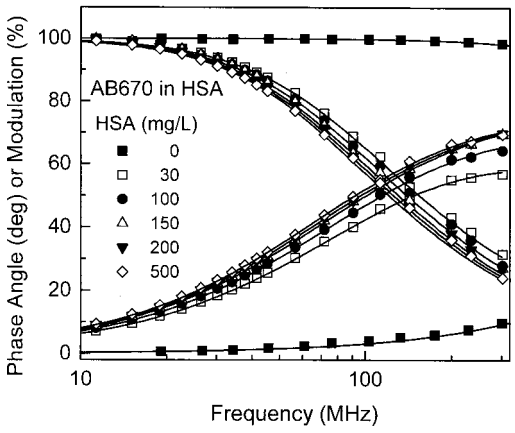

Fig. 5 Phase angle and modulation plots of 3 μM AB670 in different concentrations of HSA. Excitation was at 600 nm and observation at 670 nm.

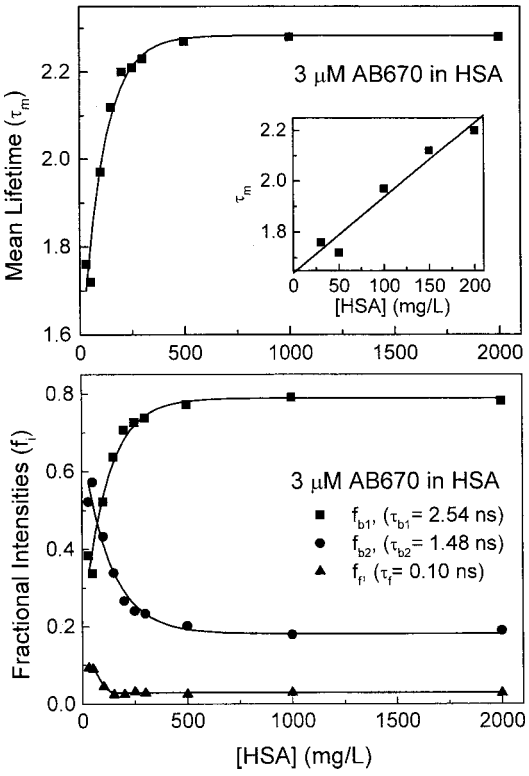

Fig. 6 Variation of the mean lifetime ($\bar{\tau}$) of 3 μM AB670 (top) and corresponding fractional intensities (f_i) (bottom) with different concentrations of HSA. The terms f_{b1}(■) and f_{b2}(●) represent the fraction intensities of bound AB670 while f_f(▲) represents the fraction intensity of the free dye in solution.

6 Anisotropy-Based Sensing of HSA Using AB670

We also determined HSA concentrations in PBS with and without RBCs using anisotropy measurements of AB670. This experiment mimicked anisotropy measurements in plasma and blood using AB670. For this, we took advantage of the high anisotropy values for AB670 in plasma and whole blood.

Figure 7 shows the anisotropy plots for AB670 in HSA (top) and HSA/RBCs (bottom). We observe that, as the con-

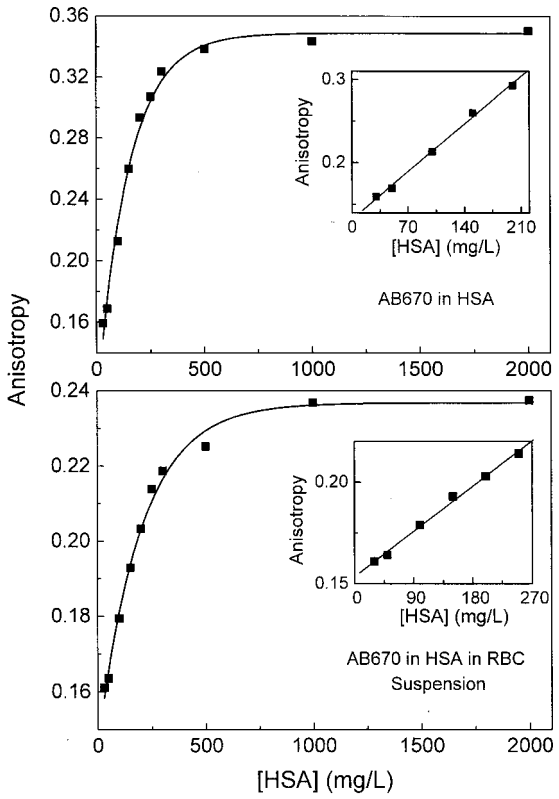

Fig. 7 Variation of the anisotropy of 3 μM AB670 with HSA in PBS (top) and in 10% Hct red blood cell suspension (bottom). Insets show expanded HSA scale in the low concentration regions. Excitation was at 650 nm and observation at 678 nm.

centration of HSA increases, AB670 anisotropy also increases, until it levels off both in the HSA and HSA/RBCs media. In both media, we observe a linear increase in the concentration range of about 30–250 mg/l. This indicates that it is possible to monitor HSA in this concentration range in both media or similar media like plasma and blood using AB670 based anisotropy measurements.

7 Discussion

We have characterized the fluorescent spectral properties of the two red emitting albumin blue dyes, AB633 and AB670, to show the feasibility of using them for fluorescent measurements directly in plasma and whole blood. We have shown that these dyes, previously reported to be specific for HSA in urine samples,[2] also bind selectively to HSA in both plasma and blood. This makes them potentially useful for the sensing of HSA both in plasma and in blood.

Recently there have been trends to develop lifetime-based methods[14–16] for sensing of analytes, since they have numerous advantages over the common intensity-based sensing methods. Unlike intensity measurements, lifetime measurements are mostly independent of probe concentrations, photobleaching, and intensity changes due to light loss. They are also less sensitive to changes in light scattering and/or absorption characteristics of samples, as has been shown in this study. Intensity-based sensing had been previously used to demonstrate the utility of AB633 and AB670 for the sensing of HSA.[2] In this study, we further demonstrate the use of lifetime-based sensing for the measurement of HSA using AB670. AB670, rather than AB633, was used for this purpose because of its larger lifetime response. We also used anisotropy measurements to monitor the concentrations of HSA in PBS and PBS/RBCs in order to mimic measurements in plasma and blood. Our results also suggest the possibility of monitoring HSA directly in plasma and blood with AB670 using anisotropy based measurements.

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