Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics

Supplementary Infomation

Revealing the ionization ability of binding site I in human serum albumin using 2-(2′-hydroxyphenyl)benzoxazole as a pH sensitive probe

Osama K. Abou-Zied*

Department of Chemistry, Faculty of Science, Sultan Qaboos University, P.O. Box 36, Postal Code 123, Muscat, Sultanate of Oman

* Tel.: (+968) 2414-1468; fax: (+968) 2414-1469. E-mail: abouzied@squ.edu.om.

Förster's resonance energy transfer

The distance between the donor (Trp214) and acceptor (HBO) can be calculated according to Förster's theory for resonance energy transfer (FRET).[1-3] The efficiency of energy transfer, E, is related to the distance (R_{DA}) between the donor and acceptor by

$$E = \frac{R_0^6}{R_0^6 + R_{DA}^6} = 1 - \left(\frac{F}{F_0}\right) \quad (1)$$

where R_0 is the Förster distance (critical distance) when the efficiency of energy transfer is 50%. F and F_0 are the fluorescence intensities of HSA in the presence and absence of the probe, respectively. The value of R_0 can be calculated from

$$R_0 = 0.211(\kappa^2 d^{-4} \phi_D J)^{1/6} \quad (2)$$

where κ^2 is the spatial orientation factor between the emission dipole of the donor and the absorption dipole of the acceptor, d is the refractive index of the medium, ϕ_D is the fluorescence quantum yield of the donor, and J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor (Figure S1) and is given by

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 \frac{\Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (3)$$

where $F(\lambda)$ is the fluorescence intensity of the donor at wavelength λ, and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. In the present case, the value of ε is 14250 $M^{-1}cm^{-1}$ for HBO.

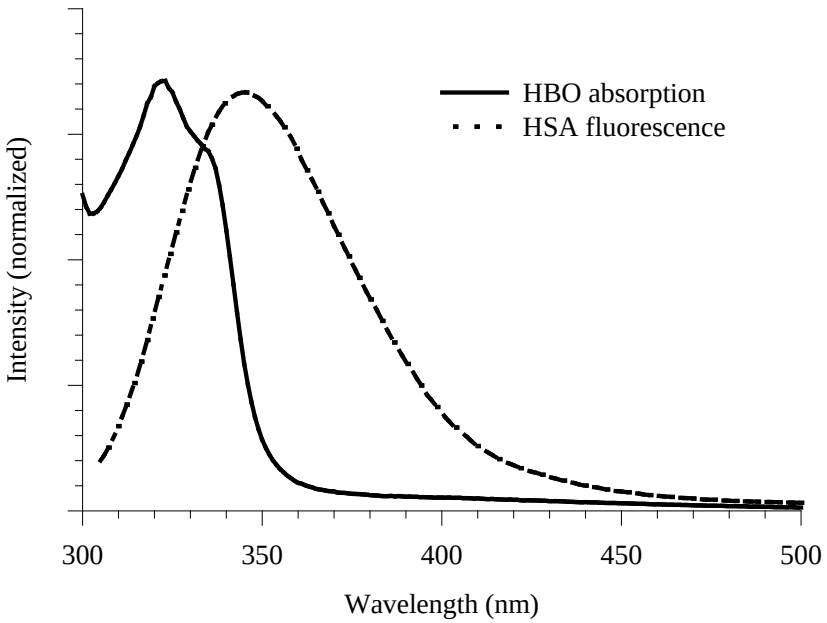

Fig. S1 Overlap of the fluorescence spectrum of HSA (λ_{ex} = 295 nm) with the absorption spectrum of HBO. The molar absorption coefficient value for HBO was: ε_{323} = 14250 $M^{-1}cm^{-1}$. Solutions were prepared in 50 mM phosphate buffer of pH 7.2. The concentration of HSA and HBO was 0.1 mM.

J can be evaluated by integrating the overlapped portion of the spectra in Figure S1. If both the donor and acceptor are tumbling rapidly and are free to assume any orientation, then the dipole orientation factor, κ^2, can adapt the value 2/3.[2,3] The value of κ^2 represents a major factor in the analysis of the energy transfer efficiencies. This factor reflects the angle between the emission transition dipole of the donor and the transition absorption dipole of the acceptor. Depending upon the relative orientation of donor and acceptor this factor can range from 0 to 4. For head-to-tail parallel transition dipoles $\kappa^2 = 4$, and for parallel dipoles $\kappa^2 = 1$. Since the sixth root is taken to calculate the distance, variation of κ^2 from 1 to 4 results in a 26% change in R_{DA}. Compared to $\kappa^2 = 2/3$, the calculated distance can be in error by no more than 35%. However, if the dipoles are oriented perpendicular to one another, $\kappa^2 = 0$, which would result in serious errors in the calculated distance. A detailed discussion about this parameter is presented in references 3 and 4.

In the present case, $d = 1.36$ and $\phi_D = 0.15$.[5] Using the aforementioned parameters, we calculated the values summarized in Table S1 for 1:1 molar ratio of HSA:HBO (0.1 mM each). The estimated donor-to-acceptor distance is less than 70 Å, indicating a major contribution from a static quenching interaction between the donor and acceptor according to Förster's nonradiative energy transfer theory.[1-3]

Table S1. Calculated parameters for the HSA:HBO complex using FRET theory.

Efficiency of Energy Transfer (%*E*)	75
Donor-Acceptor Critical Distance (R_0 (Å))	27
Donor-Acceptor Apparent Distance (R_{DA} (Å))	22
Energy Transfer Rate Constant (k x 10^{-8} (s^{-1}))	4.5

Equilibrium binding constant

Figure S2 shows the change in the HSA fluorescence intensity as a function of HBO concentration. For a small molecule such as HBO that binds to a set of equivalent sites (*m*) in a macromolecule such as HSA, we can write the following equilibrium:[6]

$$\mathrm{HSA} + m\mathrm{HBO} \overset{K_{eq}}{\rightleftharpoons} \mathrm{HSA{:}(HBO)}_m \qquad (4)$$

In this equation, $K_{eq} = [\mathrm{HSA{:}(HBO)}_m]/[\mathrm{HSA}][\mathrm{HBO}]^m$, where K_{eq} is the equilibrium binding constant for the formation of the complex $\mathrm{HSA{:}(HBO)}_m$. An expression relating the relative concentrations to the observed fluorescence (F_{obs}), and to the fluorescence of the complex ($\mathrm{HSA{:}(HBO)}_m$) and that of free HSA (F_{HSA}) can be written as:[6-11]

$$\frac{[\mathrm{HSA:HBO}_m]}{[\mathrm{HSA}]} = \frac{(F_{obs} - F_{HSA})}{(F_{\mathrm{HSA:HBO}_m} - F_{obs})} \qquad (5)$$

Using equation 2 to rewrite the equilibrium constant, one obtains the following expression:

$$F_{\text{obs}} = \frac{F_{\text{HSA}} + F_{\text{HSA:HBO}_m} K_{\text{eq}}[\text{HBO}]^m}{1 + K_{\text{eq}}[\text{HBO}]^m} \qquad (6)$$

Equation 6 represents a binding isotherm between species in equilibrium. The measured change in the fluorescence intensity of HSA as a function of HBO concentration is displayed in Figure S3. The best fit to Equation 6 is also shown. The calculations from the best fit show that m = 1 and K_{eq} = (3.5 ± 0.3) x 10^4 M^{-1}. The value of m indicates that HBO is located in one binding site. This result, along with the quenching result, indicate that HBO binds only in site I of HSA within the limit of the concentration used here (1:1 molar ratio of HSA:HBO).

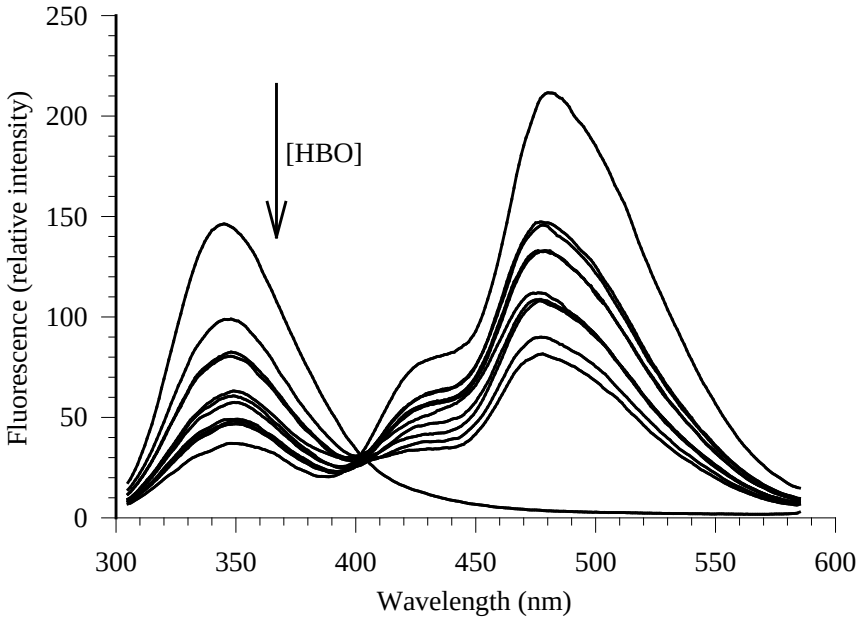

Fig. S2 Fluorescence of HSA as a function of HBO concentration. The concentration of HSA was kept constant at 0.1 mM in 50 mM phosphate buffer of pH 7.2. λ_{ex} = 295 nm.

Stern-Volmer quenching rate constant

The quenching mechanism in the HSA:HBO complex can be described by the Stern-Volmer quenching equation[3]

$$\frac{F_0}{F} = 1 + k_q \tau_0 [\text{Q}] = 1 + K_{SV}[\text{Q}] \qquad (7)$$

where k_q is the quenching rate constant of HSA, K_{SV} is the Stern-Volmer constant, τ_0 is the lifetime of HSA without the quencher (HBO), and [Q] is the quencher concentration ([HBO]). We used our measured average lifetime of HSA of 6.49 ns after excitation at 295 nm (see the Experimental section in the paper for details). By varying the concentration of HBO and keeping the HSA concentration fixed, k_q can be obtained from the slope of the regression curve of F_0/F versus [HBO] as shown in Figure S4. The calculated value is 3.9 x 10^{12} $M^{-1}s^{-1}$. The value is greater than the maximum dynamic collisional quenching constant of various kinds of quenchers with biopolymers.[12] The results confirm the major contribution of the static quenching mechanism mentioned above.

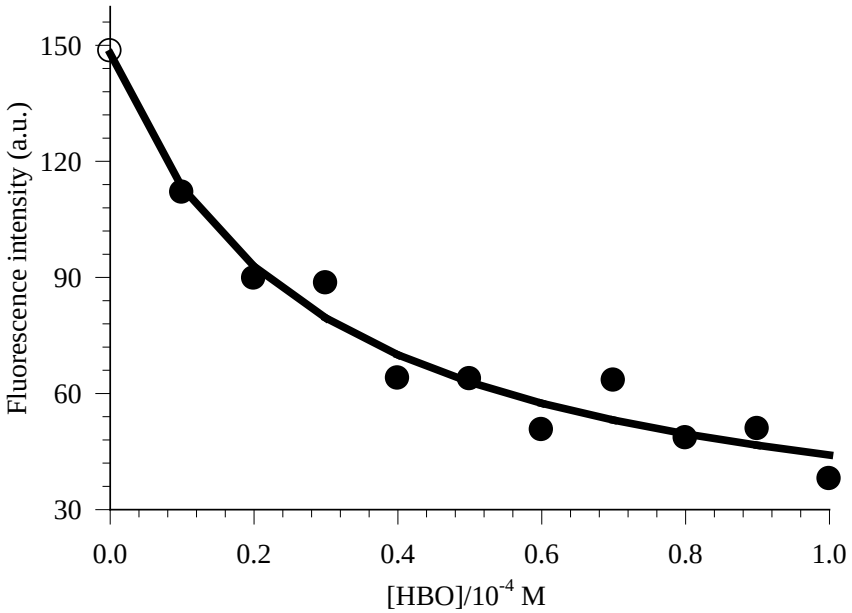

Fig. S3 Determination of the equilibrium binding constant and the number of binding sites for the HSA:HBO complex using Equation 6. The concentration of HSA was kept constant at 0.1 mM in 50 mM phosphate buffer of pH 7.2. λ_{ex} = 295 nm.

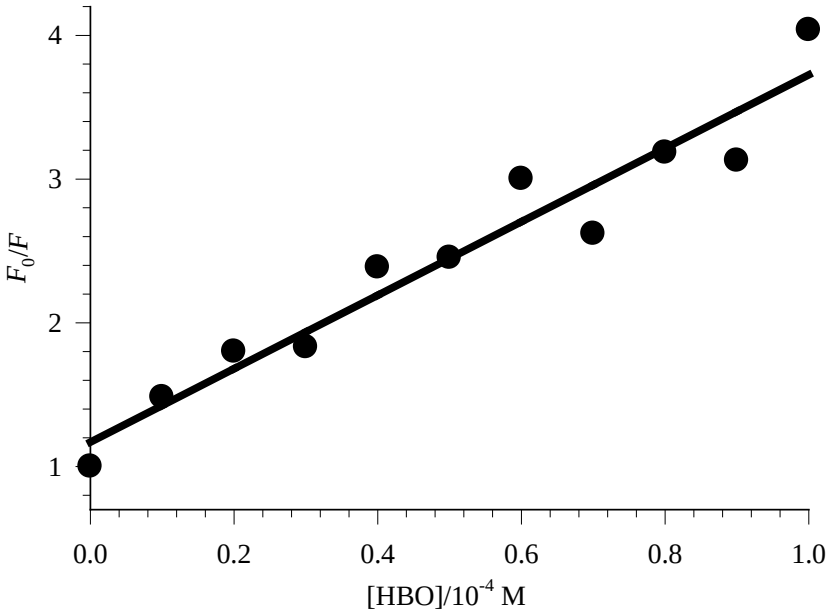

Fig. S4 Stern-Volmer plots (Equation 7) for quenching of Trp-214 fluorescence by HBO. The concentration of HSA was kept constant at 0.1 mM in 50 mM phosphate buffer of pH 7.2. λ_{ex} = 295 nm.

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