Electronic supplementary information

**Unraveling the binding interaction of Toluidine blue O with bovine hemoglobin-
A multi spectroscopic and molecular modeling approach**

Krishnamoorthy Shanmugaraj[a], Shanmugam Anandakumar[b] and Malaichamy Ilanchelian[a]*

[a]Department of Chemistry
[b]Department of Bioinformatics
Bharathiar University, Coimbatore – 641046, Tamil Nadu, India.

Elimination of inner filter effect

The inner-filter effect (IFE) refers to the absorption (or optical dispersion) of light at the excitation or emission wavelength by the compounds present in the solution.[1,2] When a ligand is added to a protein solution, if the absorption of the ligand at the excitation wavelength was strong, less light would reach the center of the solution thereby, the emission intensity of protein would be reduced. Whereas, if the added ligand posses considerable absorption at the emission wavelength of the fluorophore, it would reduce the emitted light that reaches the detector, which in turn decrease the emission intensity of protein, as well.[3] The absorption spectra of increasing concentrations of TBO are shown in (Figure S1). As depicted in Figure S1, TBO dye shows a considerable absorption at the excitation wavelength of BHb protein (295 nm). It can be seen from Figure S1, it can be emphasized that the addition of increasing concentration of TBO would significantly influence the emission spectral studies of BHb-TBO system through IFE. Thus, in the present investigation, we have carried out the emission titration experiments at low concentration of TBO (2.40 to 24.00 × 10^{-7} mol dm^{-3}) to eliminate the influence of IFE in emission spectral studies. It is pertinent to note that at low concentration regime, the absorption value of TBO is less than 0.1 at both excitation and emission wavelengths. However, it has been

reported earlier that an increment in absorption value of 0.03 corresponds to 3% decrease in emission intensity.[2]
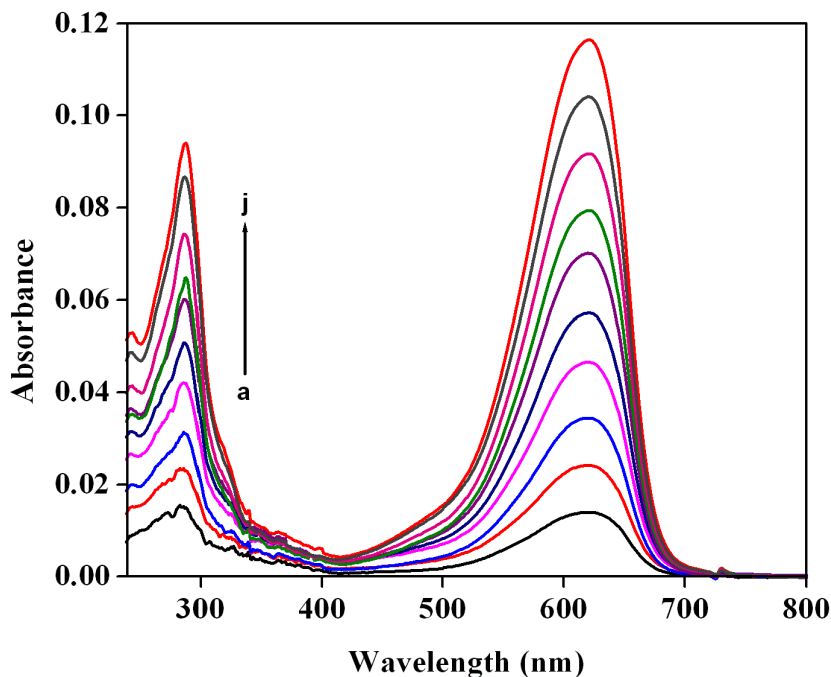

Figure S1. Absorption spectra of various concentrations of various concentrations of TBO. [TBO]: [a] 2.40×10^{-7}, [b] 4.80×10^{-7}, [c] 7.20×10^{-7}, [d] 9.60×10^{-7}, [e] 12.00×10^{-7}, [f] 14.40×10^{-7}, [g] 16.80×10^{-7}, [h] 19.20×10^{-7}, [i] 21.60×10^{-7} and [j] 24.00×10^{-7} mol dm^{-3}

As a result, the observed emission intensity has to be corrected to account for IFE before conducting an analysis of the quenching mechanism. The correction of emission intensity can be achieved by measuring the absorbance value at the excitation and emission wavelength for each concentration of TBO and then multiplying the observed emission intensity value[1-3] using the following equation (Eq. (1)).

$$F_{cor} = F_{obs} \times \text{antilog} \left[\frac{(A_{ex} + A_{em})}{2} \right] \tag{1}$$

where, F_{cor} and F_{obs} are the corrected and observed emission intensities, A_{ex} and A_{em} are the absorption values of the TBO at the excitation (295 nm) and emission (334 nm) wavelengths, respectively.

The plot of normalized emission intensity (F/F_0) of both corrected and observed emission intensities *vs* increasing concentrations of TBO are shown in Figure S2. The F/F_0 values of corrected and observed emission intensity showed a decrease with increasing concentrations of TBO and the former was slower than the latter, suggesting that the emission quenching of BHb was partly caused by the IFE of TBO. However, an evident reduction in F/F_0 values for corrected emission intensity was still observed even after the removal of IFE, which could be caused by either collisional or binding-related quenching.[3]
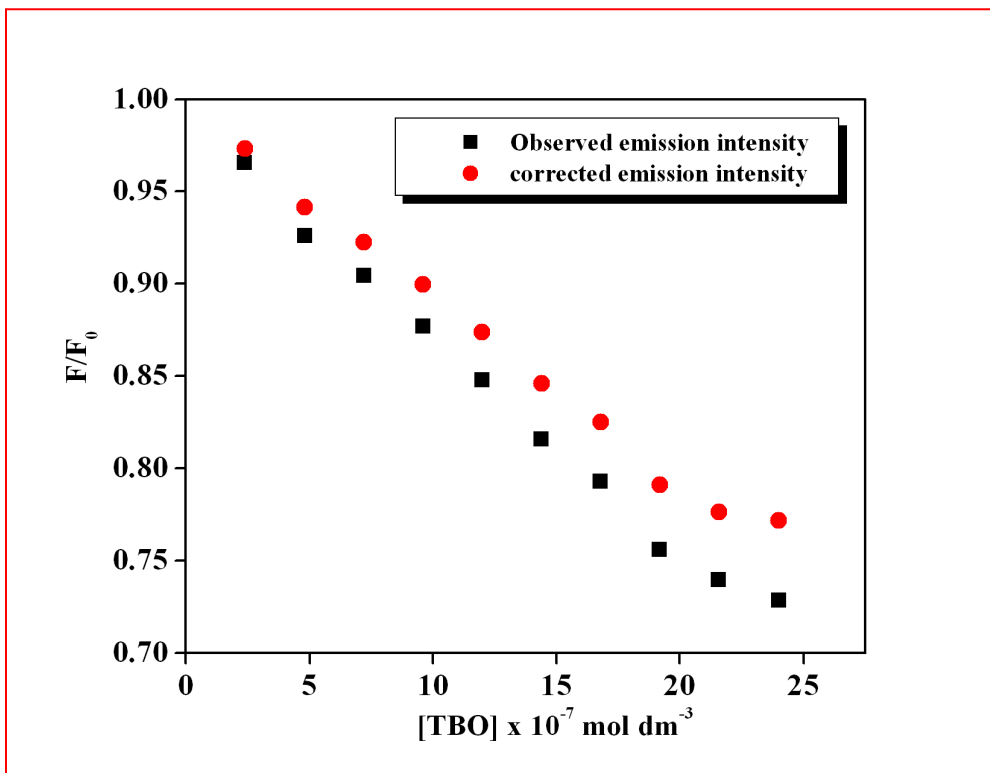
Figure S2. The effect of the inner-filter effect on the normalized emission (F/F_0) of BHb with the increasing concentrations of TBO. [TBO] = 2.40 to 24.00 × 10^{-7} mol dm^{-3}. [BHb] = 3.00 × 10^{-6} mol dm^{-3}.

Binding stoichiometry from Job's Plot
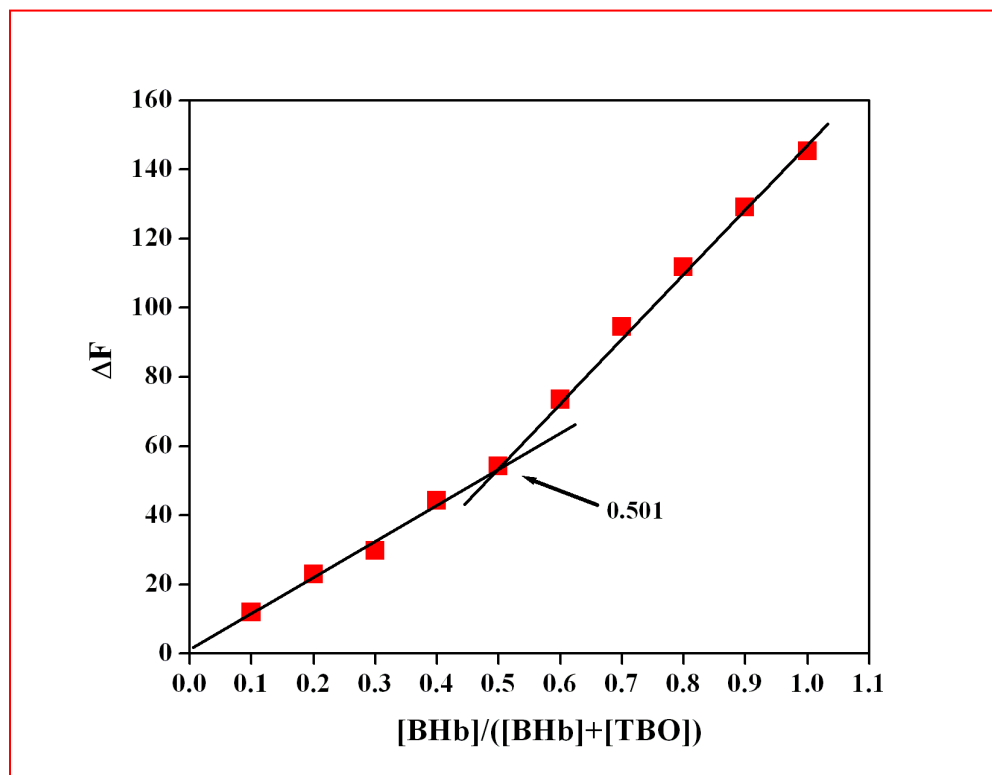
Figure S3. Job's plot for the binding of TBO with BHb. Emission intensity was recorded at 334 nm with an excitation wavelength of 295 nm.

References

1 J. R. Lakowicz. *Principles of fluorescence spectroscopy*, third ed. Springer Science+Business Media, *New York,* 2006.

2 M. Van de Weert and L. Stella. *J. Mol. Struct.* 2011, **998**, 144-150.

3 J. Zhang, Z. Tian, L. Liang, M. Subirade and L. Chen. *J. Phys. Chem. B*. 2013, **117**, 14018-14028.