Supporting Information

## Temperature Dependent Simultaneous Ligand-binding in Human

Serum Albumin

Sudarson Sekhar Sinha, Rajib Kumar Mitra and Samir Kumar Pal\*

Unit for Nano Science & Technology, Department of Chemical, Biological & Macromolecular Sciences, S. N. Bose National Centre for Basic Sciences, Block JD, Sector III, Salt Lake, Kolkata 700 098, India

\*Corresponding Author. Email: skpal@bose.res.in; Fax: 91 33 2335 3477.

## **Experimental methodology:**

Temperature dependent steady-state absorption and emission are measured with Shimadzu spectrophotometer (UV-2450) and Jobin Yvon (Fluoromax-3) fluorimeter respectively with a temperature controller attachment from Julabo (F-32). DLS measurements are done with Nano S Malvern-instrument employing a 4 mW He-Ne laser ( $\lambda$ = 632.8 nm) and equipped with a thermostatted sample chamber. All the scattered photons are collected at 173° scattering angle at 298K. The scattering intensity data are processed using the instrumental software to obtain the hydrodynamic diameter (dH) and the size distribution of the scatterer in each sample. The instrument measures the time dependent fluctuation in the intensity of light scattered from the particles in solution at a fixed scattering angle. Hydrodynamic diameters (dH) of the particles are estimated from the intensity auto-correlation function of the time-dependent fluctuation in intensity. dH is defined as,

$$d_{\mu} = kT/3\pi\eta D \tag{1}$$

where k=Boltzmann constant, T=absolute temperature,  $\eta$ =viscosity and D=translational diffusion coefficient. In a typical size distribution graph from the DLS measurement, X-axis shows a distribution of size classes in nm, while the Y-axis shows the relative intensity of the scattered light. The DTA is done in a Diamond TG/DTA instrument from Perkin Elmer. The circular dichroism (CD) measurements are done in a JASCO-815 spectropolarimeter with an attachment for the temperature dependent measurements (Peltier). The secondary structural data of the CD spectra are analyzed using CDNN software.

Fluorescence transients are measured and have been fitted by using commercially available spectrophotometer (LifeSpec-ps) from Edinburgh Instrument, U.K (excitation wavelength 409 nm, 80 ps instrument response function (IRF)) with an attachment (Julabo F-32) for temperature dependent studies. The observed fluorescence transients are fitted by using a nonlinear least square fitting procedure to a function  $(X(t) = \int_{0}^{t} E(t')R(t-t')dt')$  comprising of convolution of the IRF (E(t)) with a sum of

exponentials  $(R(t) = A + \sum_{i=1}^{N} B_i e^{-t/\tau_i})$  with pre-exponential factors  $(B_i)$ , characteristic lifetimes  $(\tau_i)$  and a background (A). Relative concentration in a multi-exponential decay is finally expressed as;  $c_n = \frac{B_n}{\sum_{i=1}^{N} B_i} \times 100$ . The quality of the curve fitting is evaluated by

reduced chi-square and residual data.

To construct time-resolved emission spectra (TRES) we follow the technique described in references<sup>1,2</sup>. As described above the emission intensity decays are analyzed in terms of the multi-exponential model,

$$I(\lambda, t) = \sum_{i=1}^{N} \alpha_{i}(\lambda) \exp[-t / \tau_{i}(\lambda)]$$
(2)

where  $\alpha_i(\lambda)$  are the pre-exponential factors, with  $\Sigma \alpha_i(\lambda)=1.0$ . In this analysis, we compute a new set of intensity decays, which are normalized so that the time-integrated intensity at each wavelength is equal to the steady-state intensity at that wavelength. Considering  $F(\lambda)$  to be the steady-state emission spectrum, we calculate a set of  $H(\lambda)$  values using,

$$H(\lambda) = \frac{F(\lambda)}{\underset{\substack{0\\\\0}}{\overset{[1]}{\prod}} I(\lambda, t)dt}$$
(3)

which for multi-exponential analysis becomes,

$$H(\lambda) = \frac{F(\lambda)}{\sum_{i} \alpha_{i}(\lambda)\tau_{i}(\lambda)}$$
(4)

Then, the appropriately normalized intensity decay functions are given by,

$$I'(\lambda, t) = H(\lambda)I(\lambda, t) = \sum_{i=1}^{N} \alpha'(\lambda) \exp[-t / \tau_i(\lambda)]$$
(5)

where  $\alpha'_i(\lambda) = H(\lambda)\alpha_i(\lambda)$ . The values of I'( $\lambda$ ,t) are used to calculate the intensity at any wavelength and time, and thus the TRES. The values of the emission maxima and spectral width are determined by nonlinear least-square fitting of the spectral shape of the TRES. The spectral shape is assumed to follow a lognormal line shape <sup>2</sup>,

$$I(\bar{\nu}) = I_0 \exp\left\{-\left[\ln 2\left(\frac{\ln(\alpha+1)}{b}\right)^2\right]\right\}$$
(6)

with  $\alpha = \frac{2b(\overline{v} - \overline{v_{\text{max}}})}{b} > 1$ , where I<sub>0</sub> is amplitude,  $\overline{v}_{\text{max}}$  is the wavenumber of the

emission maximum and spectral width is given by  $\Gamma = \Delta \left[ \frac{\sinh(b)}{b} \right]$ 

The terms b and  $\Delta$  are asymmetry and width parameters respectively. The equation (6) reduces to a Gaussian function for b=0.

The Förster distance  $(R_0)$  of donor-acceptor pair is given by,

$$R_0 = 0.211 [\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \quad (in \text{ Å})$$
(7)

where  $\kappa^2$  is a factor describing the relative orientation in space of the transition dipoles of the donor and acceptor. For donor and acceptors that randomize by rotational diffusion prior to energy transfer, the magnitude of  $\kappa^2$  is assumed to be 2/3. In the present study the same assumption has been made. The refractive index (*n*) of the medium is assumed to be 1.4.  $Q_D$  is the quantum yield of the donor in the absence of acceptor. J ( $\lambda$ ), the overlap integral, which expresses the degree of spectral overlap between the donor emission and the acceptor absorption, is given by,

$$\mathbf{J}(\boldsymbol{\lambda}) = \frac{\int_{0}^{\infty} F_{D}(\boldsymbol{\lambda}) \varepsilon(\boldsymbol{\lambda}) \boldsymbol{\lambda}^{4} d\boldsymbol{\lambda}}{\int_{0}^{\infty} F_{D}(\boldsymbol{\lambda}) d\boldsymbol{\lambda}}$$
(8)

where  $F_D(\lambda)$  is the fluorescence intensity of the donor in the wavelength range of  $\lambda$  to  $\lambda$ +d $\lambda$  and is dimensionless.  $\varepsilon(\lambda)$  is the extinction coefficient (in M<sup>-1</sup> cm<sup>-1</sup>) of the acceptor at  $\lambda$ . If  $\lambda$  is in nm, then J( $\lambda$ ) is in units of M<sup>-1</sup> cm<sup>-1</sup> nm<sup>4</sup>. Once the value of R<sub>0</sub> is known, the donor–acceptor distance (**R**) can easily be calculated using the formula,

$$R^{6} = [R_{0}^{6}(1-E)]/E$$
(9)

Here E is the efficiency of energy transfer. The transfer efficiency is measured using the relative fluorescence intensity of the donor in the absence ( $F_D$ ) and presence ( $F_{DA}$ ) of the acceptor. The efficiency E is also calculated from the lifetimes under these respective conditions ( $\tau_D$  and  $\tau_{DA}$ )<sup>1</sup>.

$$\mathbf{E} = 1 - (\tau_{\mathrm{DA}} / \tau_{\mathrm{D}}) \tag{10}$$

## References

- Lakowicz, J. R. Principles of fluorescence spectroscopy; Kluwer Academic/Plenum: New York, 1999.
- (2) Horng, M. L.; Gardecki, J. A.; Papazyan, A.; Maroncelli, M. J. Phys. Chem. 1995, 99, 17311-17337.