Supporting Information

Reversible Insulin Hexamer Assembly Promoted by Ethyl Violet; pH Controlled Uptake and Release

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Experimental

Absorption spectra were recorded with a Jasco V530 UV-Vis spectrophotometer and fluorescence spectra with a Hitachi F-4500 spectrofluorimeter. The samples were excited at 550/590 nm in the steady-state measurements. The time-resolved fluorescence measurements were carried out using a time-correlated single photon counting (TCSPC) set up from IBH (U.K.). In the present work, a 636 nm diode laser (100 ps, 1 MHz repetition rate) was used for excitation, and emission was monitored at 690 nm. The fluorescence decays [I(t)] were analyzed, in general, as a sum of exponentials,[1, 2]

$$I(t) = \sum B_i \exp(-t/\tau_i) \quad (1)$$

For anisotropy measurements, samples were excited with a vertically polarized excitation beam and the vertically and horizontally polarized fluorescence decays were collected with a large spectral bandwidth of ~32 nm. Using these polarized fluorescence decays, the anisotropy decay function, *r(t)*, was constructed as follows [1, 2].

$$r(t) = \frac{I_V(t) - GI_H(t)}{I_V(t) + 2GI_H(t)} \quad (2)$$

$I_V(t)$ and $I_H(t)$ are the vertically and horizontally polarized decays, respectively, and G is the correction factor for the polarization bias of the detection set up.[1] Kinetic analysis of the anisotropy decay provides the rotational correlation time constant, τ_r According to the Stokes-Einstein relationship (eq.3),[3] τ_r is related to the rotational diffusion coefficient (D_r) and the hydrodynamic molecular volume (V),

$$\tau_r = \frac{1}{6D_r} \quad \text{where} \quad D_r = \frac{RT}{6V\eta} \quad (3)$$

where, η is the viscosity of the medium and T is the absolute temperature. Experimentally τ_r can be evaluated from the anisotropy decay obtained as per equation 3.

The dynamic light scattering (DLS) measurements were carried out using Malvern 4800 Autosizer employing an Ar ion laser (λ=514.5 nm) and digital correlator. The scattered light intensity was monitored at a scattering angle of 130° and the intensity correlation function over a time range of 10^{-6} to 1s was computed. Circular Dichroism (CD) studies were carried out on a MOS-500 spectrometer from BioLogic, SAS, France. The spectra were measured in the wavelength range 200−400/800 nm using a quartz cuvette with 1 mm path length.

The ^{1}H-NMR experiments were performed in D_2O (99.8%) using a Bruker Avance 500 MHz spectrometer at TIFR, Mumbai, India and the molecular docking calculations were done with AutoDock suite 4.2.6.

Human insulin sample was generously supplied by USV Pvt. Ltd, Mumbai, India and was used as received. All triphenylmethane dyes, Ethyl Violet (EV), Crystal Violet (CV) Malachite Green (MG) and Brilliant Green (BG) were obtained from Aldrich and were used as received. Nanopure water (Millipore Gradient A10 System; conductivity of 0.06 μS cm^{-1}) was used to prepare the sample solutions. All measurements were performed at pH 7.4 (in 10mM Tris buffer) at ambient temperature (24±1ºC), unless specified. Aqueous solution of native human insulin was prepared by dissolving it in slightly acidic conditions, below pH 5 and the pH was adjusted to 6.4, which gave a clear solution. Further dilutions were carried out with Tris buffer solutions maintained at pH 7.4.

Stock solution of human insulin was prepared by dissolving 6 mg/ml of insulin in slightly acidic aqueous solution, below pH 5 and the pH was adjusted to ~6, which gave a clear solution. In a solution free of metal ions, especially Zn^{2+}, insulin exists as monomer at lower pH, and is dimeric in the pH range 5-8.[4] However, in the presence of Zn^{2+} the dimers form the hexamer assembly, and is stable in the pH region 5-8.[4]

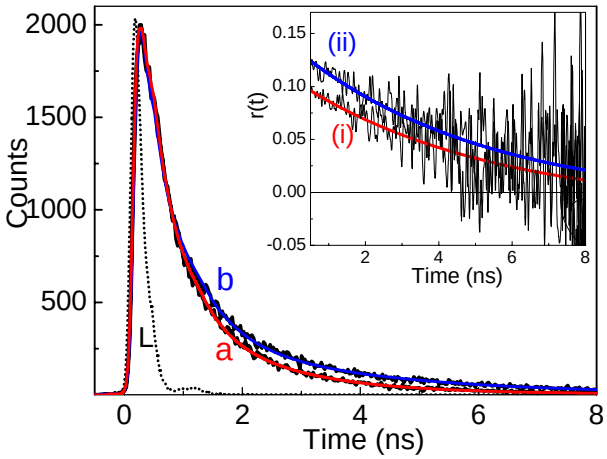


Figure S1. Fluorescence decay traces recorded at 690nm (λ_{ex} 636nm) from EV (2μM) solution containing (a) insulin (150μM) and (b) insulin (150μM) and Zn^{2+} (100 μM). L represents the excitation lamp profile (λ_{ex} 636 nm, λ_{mon} 690 nm). Inset: Anisotropy decay traces recorded from the above solution **a** (i) and solution **b** (ii).

Table S1: Fluorescence lifetime and Anisotropy values for EV (~2μM) in Tris buffer at pH 7.4, in presence and absence of insulin and Zn^{2+}. λ_{ex} 636 nm, λ_{mon} 690nm.

Insulin (μM)	Zn^{2+} (μM)	Lifetime Values			Anisotropy
		τ_1(ns) (a_1%)	τ_2(ns) (a_2%)	τ_{av} (ns)	τ_r (ns)
0	0	<.001	---	<.001[#]	---
180	0	0.40 (40)	1.20 (60)	0.88	5.12
180	100	0.51 (47)	2.51 (53)	1.57	5.29

[#]from ref [5]

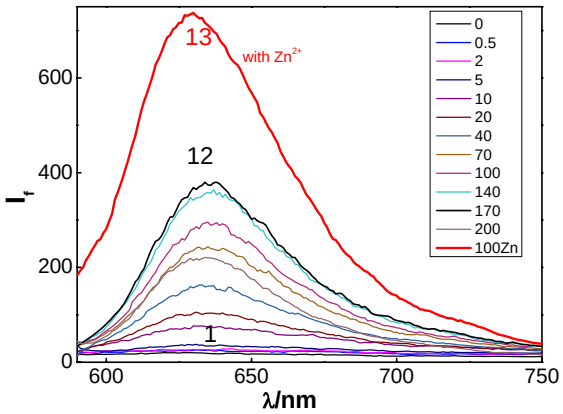


Figure S2. Emission spectra of CV-Insulin in the absence and presence of Zn^{2+}.

Note S1: ^{1}H-NMR spectroscopic studies were performed to look into the overall spectral changes of insulin in absence and in presence of EV, CV and the known hexamer inducing metal ion, Zn^{2+}. In absence of the external agents the ^{1}H-NMR spectra of insulin displayed the multiple overlapping resonances, both in the aliphatic and aromatic regions as shown in Figure S3. However, we observed the amide resonances at δ 7.6 and 7.7 which clearly indicate the presence of monomeric units of insulin at the experimental pH. Addition of Zn^{2+} ions to insulin solution resulted in the decrease of signal intensity but many well resolved resonances appeared in the aliphatic region of the spectrum. Iinsulin in presence of EV, displayed very similar changes in the spectral features in aliphatic as well as aromatic region of the spectrum. It was interesting to observe that in presence of Zn^{2+} or EV, the amide resonances disappeared indicating aggregation of monomeric insulin units. On the other hand, in presence of CV, ^{1}H-NMR spectra of insulin did not show any change in the spectral features as compared to free insulin under similar experimental conditions. This fact clearly demonstrates that unlike EV or Zn^{2+}, the CV dye could not induce insulin aggregation in to the hexameric assembly. To elucidate the exact positining of EV and such distinctive behaviour of structurally similar molecules, advanced biomolecular NMR spectroscopic

studies with isotopically labelled insulin need to be carried out. Such detailed studies will be attempted in collaboration with expert groups in the due course.

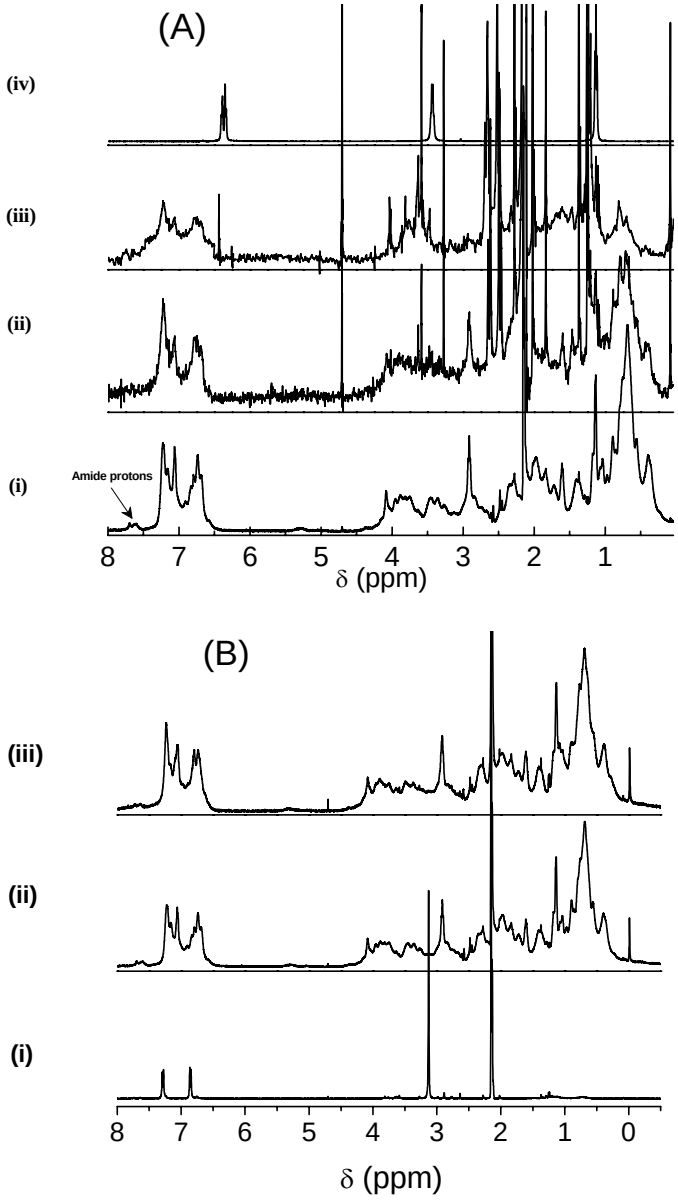


Figure S3 (A) ^{1}H-NMR spectra of Insulin (500µM) (i); Insulin with EV (137µM) (ii); Insulin with Zn^{2+}(200µM) (iii) and EV (100µM) (iv) in D_2O at pH 6.5. (**B**) ^{1}H-NMR spectra of CV (100µM) (i); Insulin (500µM) (ii) and Insulin with CV (240µM) (iii) in D2O at pH 6.5.

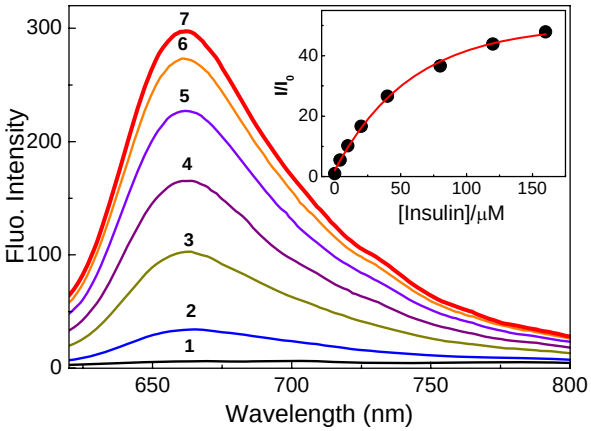
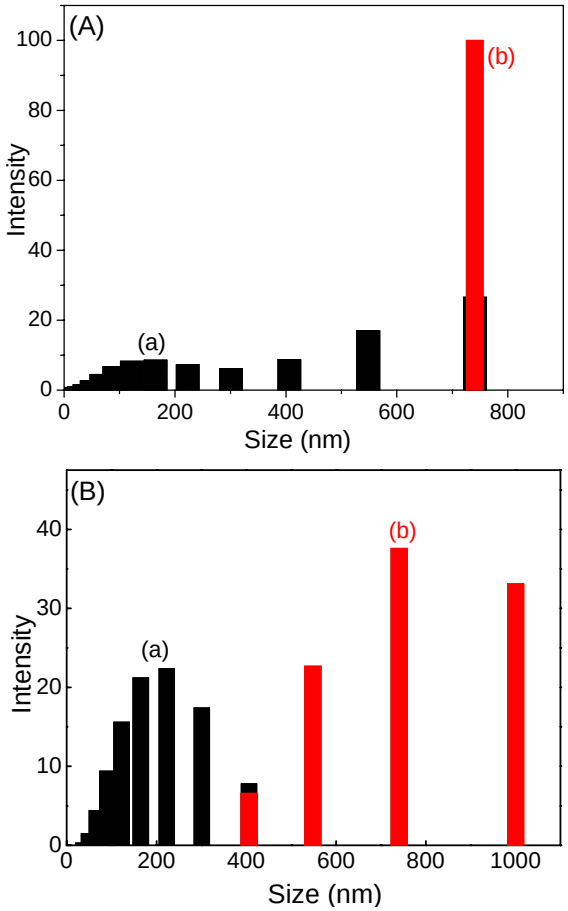


Figure S4. Fluorescence enhancement observed for Brilliant Green (BG) in solutions containing Zn^{2+} and [Insulin]/μM; 1) 0; 2) 4; 3)20; 4) 40; 5) 80; 6) 120; 7) 160. Inset shows the relative enhancement plotted against [Insulin] showing 48 fold increase in the fluorescence. No enhancement was seen in the presence of insulin alone.



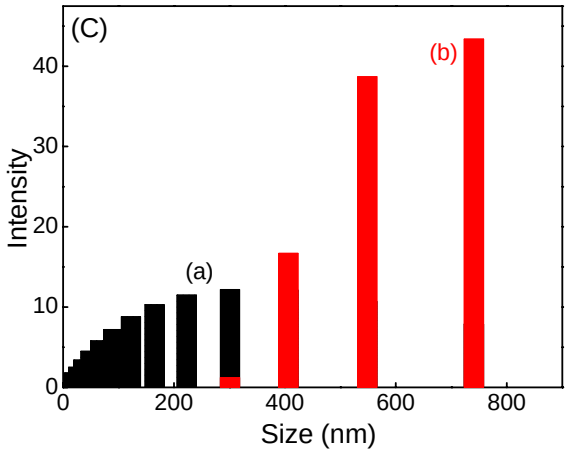


Figure S5. Scattering intensity profile recorded from an aqueous solution of (i) insulin and the TPM dye (A) CV, (B) BG and (C) MG. (a) insulin (150μM) with the TPM dye (8μM) and (b) insulin-dye containing Zn^{2+}(100μM).

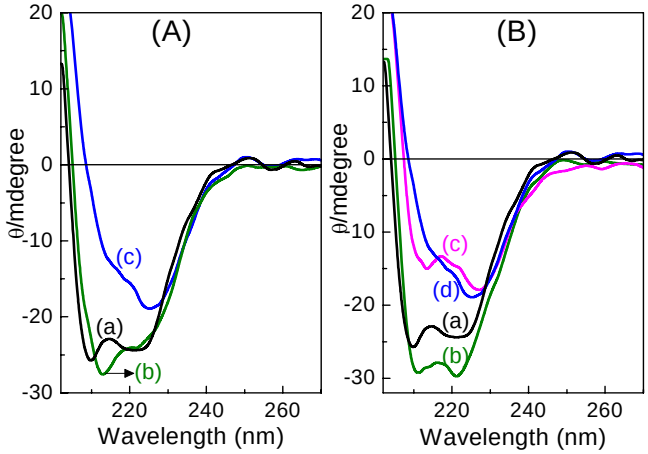


Figure S6. (A) CD spectra recorded from insulin alone (50μM) (a); insulin with Brilliant Green (BG, 20μM) (b); (3) insulin with Zn^{2+} (150μM) (c). (B) CD spectra recorded from insulin alone (50μM) (a); insulin with Malachie Green (MG, 20μM) (b); insulin solution containing Crystal Violet (CV,100μM) (c) and insulin with Zn^{2+} (150μM) (d).

Note S2: In supporting the interaction of EV with the three dimer helices, we carried out molecular docking calculations using AutoDock 4.2.6 suite at a very preliminary level.[6] Among the few docked structure obtained with insulin hexamer and EV coordinates, some of them provided stabilized configuration with EV dye incorporated near to the central binding

core (Figure S7). However, we believe that these results need to be refined with taking care all the parameters, such as solvent contributions and allowing the formation of in situ hexamer assembly. These advanced calculations are being attempted and will be reported separately.

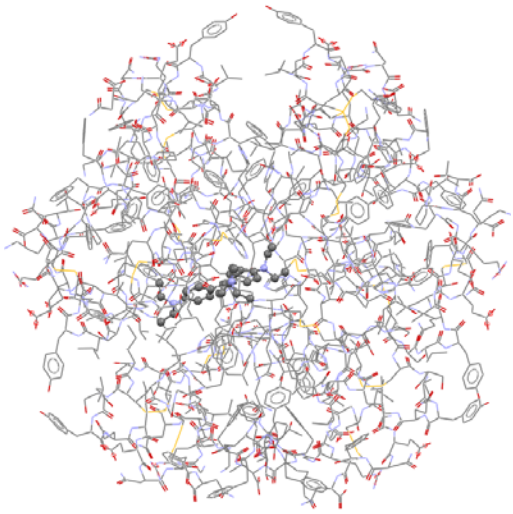


Figure S7. Preliminary out put stucture of molecular docking (AutoDock 4.2.6) calculation with Insulin Hexamer (pdb file 4aiy.pdb) and EV dye. Hydrogens are removed for clarity.

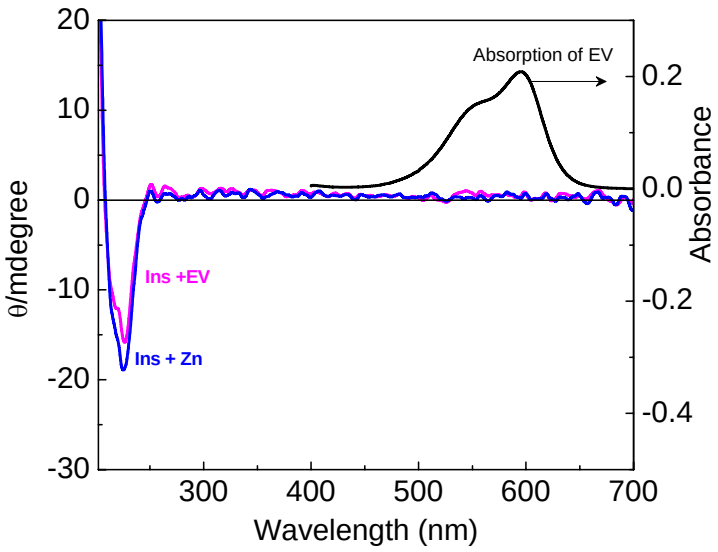


Figure S8. Full CD spectra recorded for insulin-Zn and Ins-EV systems and the absorption spectrum of EV displaying its region of steady-state absorption. No induced CD (ICD) bands were seen as discussed in the main text.

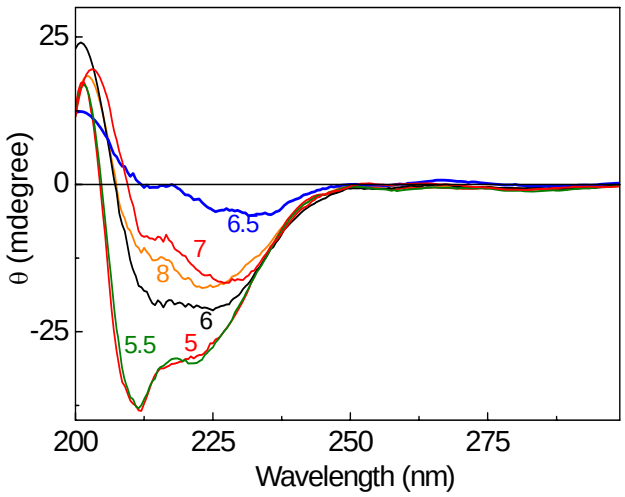


Figure S9: CD spectra recorded for the EV-Insulin system at different pHs.

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[6] Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comput. Chem.* **1998**, *19*, 1639−1662.