Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Electronic Supporting Information

SYPRO Orange-a new gold standard amyloid probe

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Materials and Methods

Bovine insulin, hen egg white lysozyme (HEWL), bovin serum albumin (BSA), calf thymus (CTDNA), ThT, SYPRO Orange and NaCl were purchased from Sigma-Aldrich. ThT was purified before use as mentioned earlier.[1] High purity water with resistivity of 18.2 MΩ cm (25^0C) was used for the preparation of aqueous solution. All experiments were performed with 2μM of SYPRO Orange.

To prepare insulin fibril, bovine insulin (2mg/ml) in 20% acetic acid solution was heated at 70 °C under vigorous stirring for 24 h.[2] To prepare insulin fibril at physiological pH (7.4), the acidic solution of fibril was diluted 12 times with water and pH was adjusted by adding dilute sodium hydroxide solution. Fibrillation of insulin at pH 7.4 was carried out by dissolving 2mg/ml protein in 20% acetic acid and dilute sodium hydroxide was added to change its pH to 7.4. The protein solution was heated at 37 ^{o}C for 24 h under constant stirring to get the fibrils.[3] To prepare lysozyme fibrils, hen egg white lysozyme (HEWL, 1.5 mg/ml) was dissolved in aqueous hydrochloric acid solution of pH 1.7. The solution was heated at 65 ^{o}C with constant stirring for 4 h.[4] The fibrils solution at physiological pH was prepared by adding sodium hydroxide solution to the acidic fibril solution. Bovine serum albumin (BSA) fibrils were prepared by incubating 2 mg/ml in 10 mM Tris-HCl buffer (pH 7.4) at 65 ^{o}C without agitation for 4 h.[5] The formation of fibrils was confirmed through ThT assay.[5] Highest concentration of insulin fibrils used in the present study was 30 μM due to its limited solubility in solution with pH=7.4.

The absorption and steady-state emission spectra were recorded using JASCO spectrophotometer (model V-650) and Hitachi spectrofluorometer (model F4500), respectively. The emission quantum yields (ϕ) were measured by comparative method[6] using Nile red in dioxane (ϕ=0.7) as reference.[7] Time-resolved emission studies were carried out using a time

correlated single photon counting (TCSPC) based spectrofluorometer from IBH, UK as described earlier.[8] Samples were excited by 445 nm laser diode and emission was collected at magic angle condition.[9] The instrument response function (IRF) of TCSPC setup was ~160 ps. All spectroscopic studies were performed in thermostatic optical cell with temperature of 25±1^{o}C.

Quantum chemical calculations were performed using Gaussian 03 packages.[10] The ground state structure of SYPRO Orange was optimized by DFT method using B3LYP functional and 6-31++g (d,p) basis function.[11, 12] The contribution of the solvent (water) was incorporated using conductor like polarizable continuum model (CPCM).[13] The optimized structure thus obtained was used for the molecular docking studies. Blind molecular docking studies were carried out using Autodock 4.0 suite as described earlier.[14] As there is no detail structural information on insulin fibrils, the fibrillar structure from the pdb file with ID of 2MVX has been selected. This fibril is made up of a variant of $A\beta_{1-40}$ peptide which is associated with the early onset of Alzheimer's diseases. The peptide corresponding to 2MVX is more prone to fibrillation and also more neurotoxic compared to its wild type variant.[15] Interaction between ligand and host molecules was predicted with the help of Discovery Studio Visualizer (version 4.1).

The binding parameters of SYPRO Orange with fibril have been estimated using following modified Benesi-Hildebrand equation.[16, 17]

$$\frac{1}{\Delta I_f} = \frac{1}{K(I_c - I_0)} \frac{1}{[fibril]} + \frac{1}{(I_c - I_0)} \quad \text{(S1)}$$

where ΔI_f represents the fibril induced changes in the fluorescence intensity, K is the association constant, I_C and Io are the fluorescence intensities of the dye when it is completely bound with fibril and free state, respectively.

The limit of detection (LOD) for probes was calculated using following equation.[18, 19]

$$LOD = \frac{3.3 * \sigma}{S} \quad \text{(S2)}$$

where σ is standard deviation of measurements and S is rate of change of emission intensity with fibril concentrations.

Emission transient decays have been fitted with multi exponential decay function and the average lifetime was calculated using the following equation.

$$\tau_{avg} = \frac{\sum a_i \tau_i^2}{\sum a_i \tau_i} \quad \text{(S3)}$$

Where a_i and τ_i are the amplitude and decay time constants of exponential decay function.

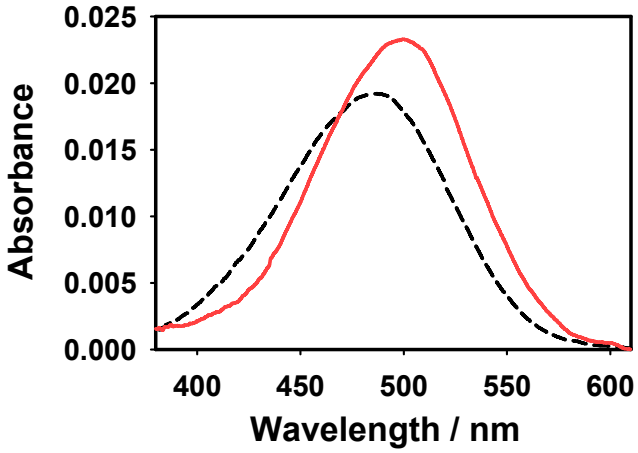

Figure S1: Absorption spectra of SYPRO Orange in water (- - - - -) and 30 μM insulin fibrils (——).

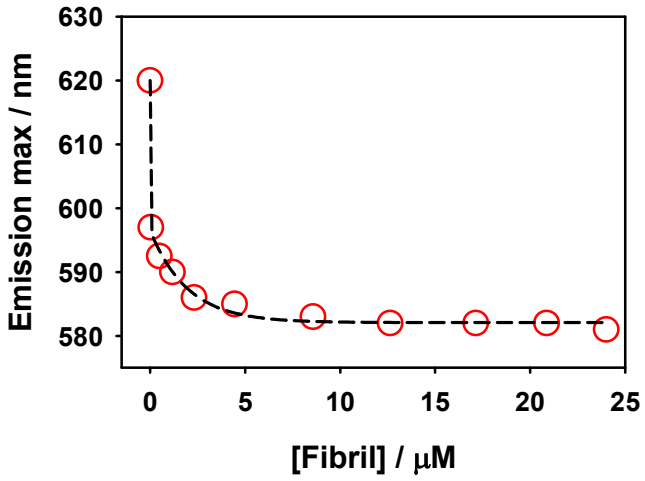

Figure S2: Variation in the emission maxima for SYPRO Orange with fibril concentration.

To check the interference from other molecules in the detection of amyloid fibrils using SYPRO Orange, its emission spectra have been recorded in presence of different biomolecules, like native proteins, DNA, etc. The enhancements in the emission intensity of SYPRO Orange due to its interaction of native protein and DNA molecules are shown in figure S3. It is evident from the figure that changes in the emission intensity of SYPRO Orange in presence of native proteins (insulin, BSA) are quite negligible. Similarly the changes in the emission intensity of SYPRO Orange in presence of very high concentration of calf thymus DNA (1 mM) is significantly less than that in dilute fibrillar solution (30 μM). These results show that SYPRO Orange is highly selective to the amyloid fibrils.

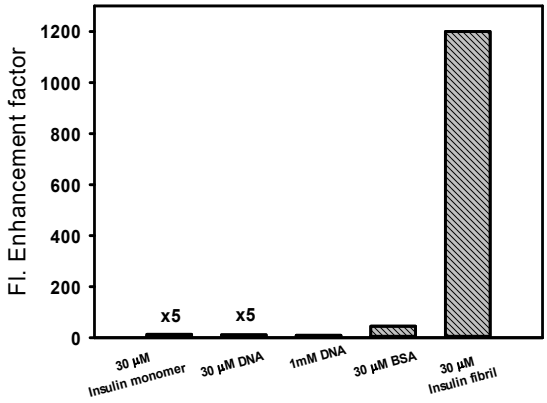

Figure S3: The bar diagram showing the fluorescence intensity enhancement due to the presence of different bio-molecular systems.

It has been reported that the fibrils prepared in the acidic solution (pH 2) and in neutral solution (pH 7.4) have quite distinct structures.[3] Different polymorphisms in these two types of fibril arise due to extensive difference in the H-bonding network.[3] To understand the effect of such polymorphisms on the sensitivity of SYPRO Orange, we have recorded the emission spectra of SYPRO Orange in amyloid fibrils prepared in solution with pH 7.4 and the results are compared with ThT (cf. figure S4). It is seen that the addition of 30 μM fibrils prepared at pH 7.4 leads to the emission enhancement of 345 and 1055 times for ThT and SYPRO Orange, respectively. Thus, like fibrils prepared in acidic solution, SYPRO Orange shows ~3 times more emission enhancement than ThT in fibrillar solution prepared at pH 7.4. This result also indicates

that irrespective of different polymorphisms in fibrils prepared by two different methods, the sensitivity of SYPRO Orange remains unchanged.

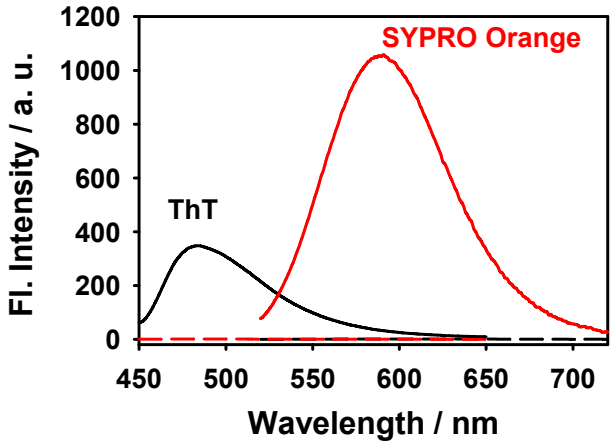

Figure S4: The emission spectra of ThT (λ_{ex}=440 nm) and SYPRO Orange (λ_{ex}=500 nm) in water (dashed) and in presence of 30 μM insulin fibril prepared at pH 7.4 (solid).

We have also examined the superior amyloid sensitivity of SYPRO Orange over ThT with fibrils from different proteins. Thus, the emission spectra of SYPRO Orange and ThT have been recorded in amyloid fibrils prepared from hen egg white lysozyme (HEWL) and bovine serum albumin (BSA) and results are shown in figure S5. Thus, the figure suggests that irrespective of the nature of protein, the amyloid sensitivity of SYPRO Orange is always better than ThT.

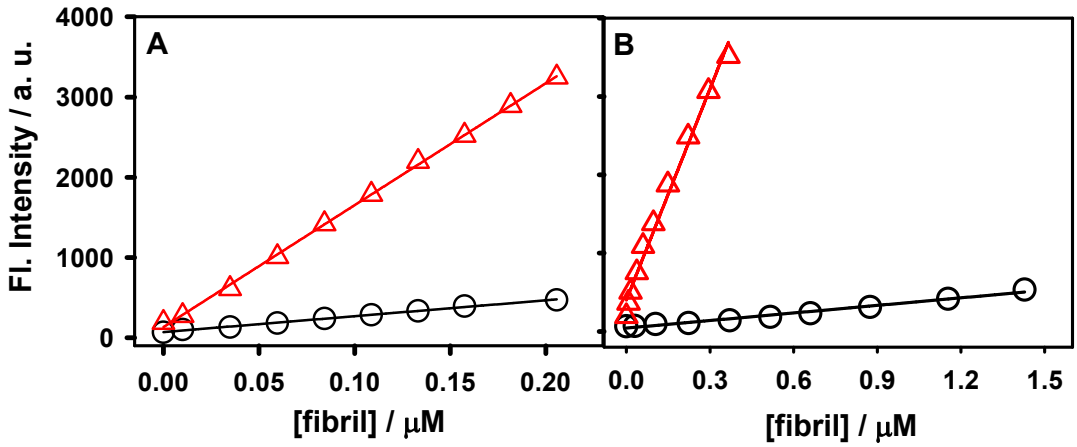

Figure S5: The variation in the fluorescence intensity of SYPRO Orange (Δ) and Thioflavin T (○) with (A) HEWL (B) BSA fibril concentration.

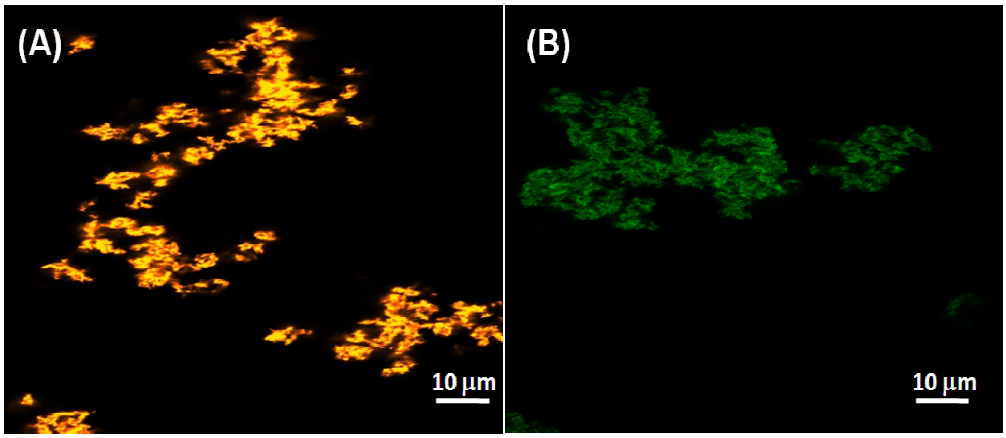

Figure S6: Fluorescence microscopic images of insulin fibril stained with (A) SYPRO Orange and (B) ThT.

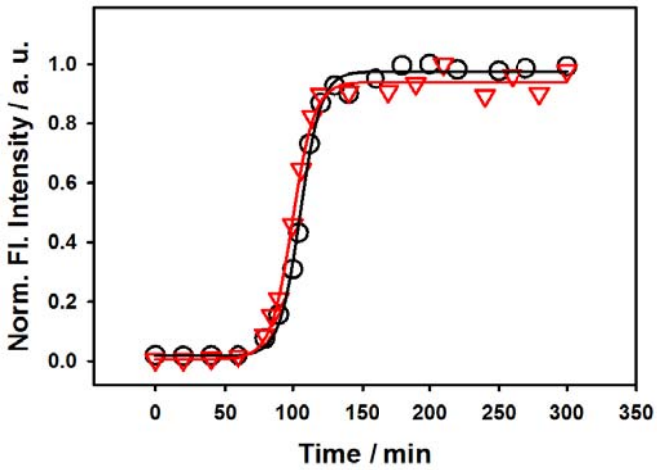

Figure S7: Fibrillation kinetics of bovine insulin (2 mg/ml) in 25 mM HCl and 100 mM NaCl monitored using SYPRO Orange (○ at 580 nm) and Thioflavin T (▽ at 490 nm).

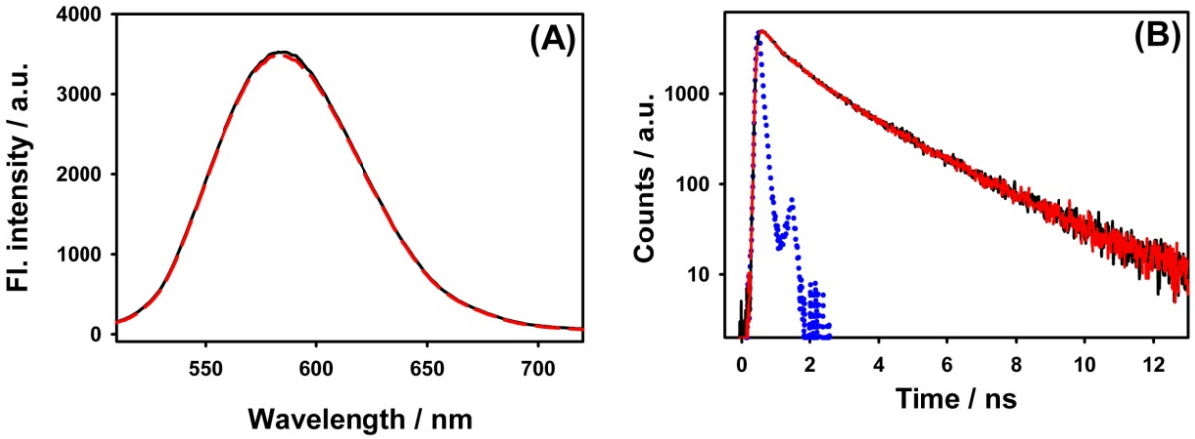

Figure S8: (A) Emission spectra and (B) emission transient decays of SYPRO Orange in fibril solution in absence (——) and presence of 500 mM NaCl (- - - -). The dotted curve in panel B represents IRF.

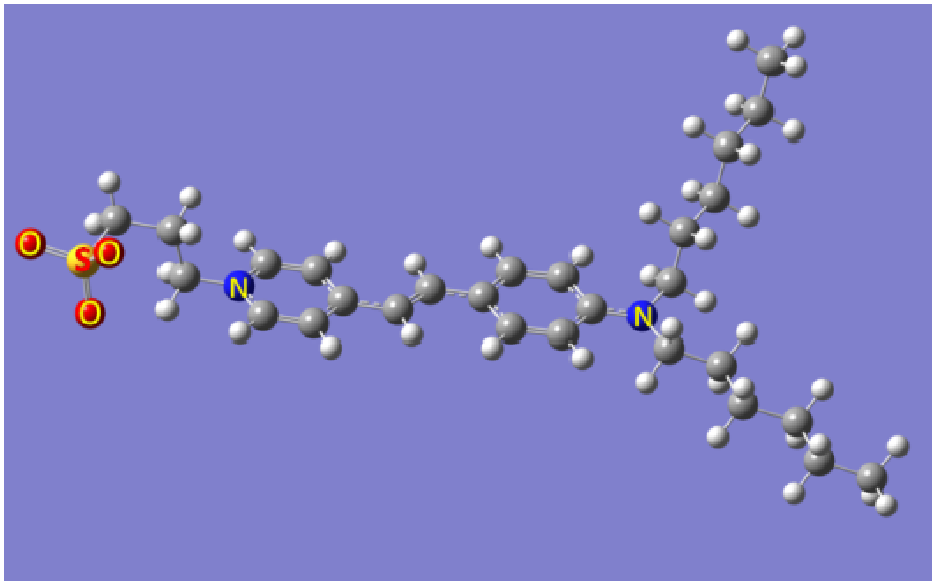

Figure S9: Optimized ground state geometry of SYPRO Orange in water calculated by quantum chemical calculations.

Table S1- Fitting parameters of fluorescence decay traces of SYPRO Orange in fibrillar solutions.

[fibril]/ μM	a_1	τ_1 / ns	a_2	τ_2 / ns	a_3	τ_3 / ns	τ_{avg} / ns*
0.04	78.4	0.03	10.1	0.31	11.5	1.90	0.27
0.08	54.7	0.03	23.1	0.32	22.2	1.83	0.50
0.24	31.8	0.05	34.9	0.37	33.3	1.91	0.78
1.18	16.1	0.09	40.1	0.51	43.9	2.15	1.16
3.04	11.7	0.14	38.9	0.66	49.4	2.31	1.41
7.06	5.8	0.12	33.9	0.71	60.2	2.38	1.68
10.0	6.9	0.18	32.9	0.85	60.2	2.50	1.80
15.0	5.4	0.17	32.6	0.89	62.0	2.58	1.90
20.9	6.3	0.24	28.5	0.94	65.3	2.58	1.96
26.7	3.5	0.21	32.1	0.94	64.4	2.62	1.99
30.0	4.4	0.20	31.5	0.94	64.1	2.63	1.99

* The average lifetime is calculated by using equation S3.

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