Electronic Supplementary Information

Early Detection of Insulin Fibrillation: Fluorescence Lifetime Assay to Probe the Pre-Fibrillar Regime

Jyotirmayee Mohanty,* Sharmistha Dutta Choudhury, Haridas Pal, Achikanath C. Bhasikuttan*

Radiation & Photochemistry Division, Bhabha Atomic Research Centre,
Mumbai 400 085, India. Fax: (+) 91-22-25505331/25519613

E-mail: jyotim@barc.gov.in (JM); bkac@barc.gov.in (ACB)

Experimental Section:

Thioflavin T (ThT), obtained from Sigma-Aldrich was purified by column chromatography using silica gel column and mildly acidic methanol as eluent. The purity was further confirmed by the ^{1}H NMR, which showed only the corresponding peaks as reported for ThT.[1] Native human insulin was from USV Ltd., India or from Sigma-Aldrich and was used as received. Nanopure water (Millipore Gradiant A10 System; conductivity of 0.06 μS cm^{-1}) was used throughout for solution preparation. The sample solution was prepared by dissolving human insulin (2mg/ml) in 25 mM of HCl, 0.1 M NaCl (pH ~1.7) and 10 μM ThT was added to this solution, as reported in the literature.[2] The net solution was incubated at 60°C. Fibril formation was monitored by recording the absorption, fluorescence spectra and fluorescence decay traces of the sample solution. At the specified time lapse, the measurements were completed within a time span of 1-2 min, so as to minimize the error on the lag time. Absorption spectra were recorded with a Shimadzu model 160-A UV-vis spectrophotometer (Tokyo, Japan). Steady-state fluorescence spectra were recorded using a Hitachi F-4500 spectrofluorimeter (Tokyo, Japan). For steady state fluorescence measurements, the samples were excited at 440 nm. Fluorescence lifetime measurements were carried out using a time-correlated-single-photon-counting (TCSPC) spectrometer (IBH, UK). In the present work, a 406 nm diode laser (~100 ps, 1 MHz repetition rate) was used for excitation and a MCP PMT was used for fluorescence detection. From the measured decay traces, the time constants were evaluated following a reconvolution procedure.[3] The fluorescence decays, I(t) were analyzed using a multi-exponential function as

$$I(t) = \sum_i B_i \exp(-t/\tau_i) \qquad (1)$$

where, B_i and τ_i are the pre-exponential factor and the fluorescence lifetime for the ith component of the fluorescence decay. The quality of the fits and consequently the mono-, bi- and tri-exponential natures of the decays were judged by the reduced chi-square (χ^2) values and the distribution of the weighted residuals among the data channels. For a good fit, the χ^2 value was close to unity and the weighted residuals were distributed randomly around zero among the data channels.[3,4]

Note-1

The probe, Thioflavin T (ThT) is known to have very fast excited state deactivation and hence low emission yield in low viscosity solvents. Due to this, with the TCSPC instrument having ~50 ps time resolution, no meaningful excited state decay parameter could be analyzed for free ThT in aqueous solution. However, on binding to the insulin aggregates/fibrils, the micro-environment around the dye radically changes (becomes more rigid) and the excited state non-radiative relaxation process, which arises due to the intramolecular torsional motion between the benzthiazole and dimethylaminobenzene groups, gets retarded. This leads to the appearance of longer lifetime component in the ThT decay profile, the contribution of which increased gradually to attain a saturation value at longer time (Table S1). The decay analysis also makes it clear that there is contribution from a different binding environment, in the very early stage, which gradually disappeared enroute to the mature fibril formation. These early time structures could be the precursor species like the high molecular weight aggregates or the globular aggregates.

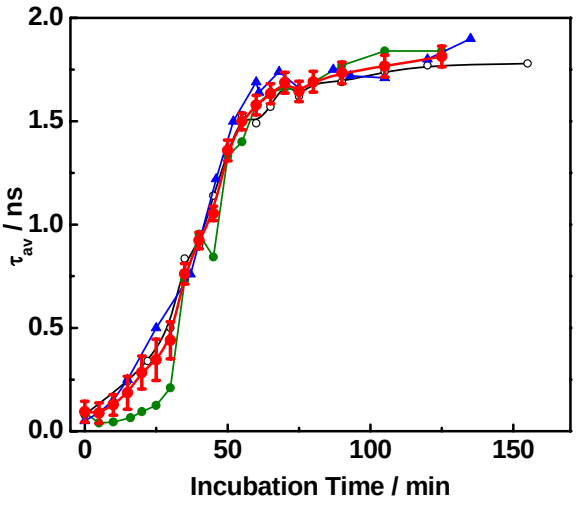

Figure S1. Figure 2 (as in main text) showing the reproducibility in the lifetime measurements. The traces (blue, green and black color) from 3 independent set of measurements and the averaged trace (red color) with error bars are shown.

Table S1: The analysis of one set of the fluorescence decay curves of ThT (10 μM) in insulin solution at pH 1.8 and 60 °C at different time intervals. The τ_{ave} is the average from 3 such independent measurements.

Incub. time (min)	τ_1*/ ps (a_1)	τ_2 /ns (a_2)	τ_3 /ns (a_3)	τ'_{ave}/ ns (single measurement)	χ^2	τ_{ave} / ns (from 3 measurements)
0	**50 (97%,)**	**1.02 (3%)**		**0.08**	**1.9#**	**0.09**
15	50 (87%,)	1.54 (13%)		0.24	1.86#	0.19
20	50 (79%,)	1.57 (21%)		0.37	1.69#	0.28
30	50 (71%,)	1.59 (29%)		0.50	1.5#	0.44
35	**50 (50%,)**	**1.62 (50%)**		**0.84**	**1.36**	**0.76**
40	50 (43%,)	1.44 (48%)	2.32 (8%)	0.91	1.35	0.92
45	**50 (30%,)**	**1.35 (48%)**	**2.17 (22%)**	**1.14**	**1.32**	**1.06**
50	50 (18%,)	1.3 (39%)	1.93 (43%)	1.35	1.3	1.36
55	50 (11%,)	1.18 (27%)	1.94 (62%)	1.53	1.27	1.50
60	50 (9%,)	1.18 (31%)	1.87 (60%)	1.49	1.25	1.58
65	**50 (5%,)**	**0.98 (15%)**	**1.78 (80%)**	**1.57**	**1.17**	**1.63**
70	50 (3%,)	1.15 (20%)	1.89 (77%)	1.69	1.08	1.69
75	50 (3%,)	0.98 (10%)	1.75 (87%)	1.62	1.16	1.64
80	**50 (3%,)**	**1.26 (16%)**	**1.84 (81%)**	**1.69**	**1.15**	**1.58**
90	**50 (3%,)**		**1.74 (97%)**	**1.69**	**1.1**	**1.73**
105	50 (3%)		1.79 (97%)	1.74	1.1	1.77
120	50 (2%)		1.80 (98%)	1.77	1.12	1.81
160			**1.78 (100%)**	**1.78**	**1.18**	**1.81**

** For smooth fitting, 50 ps component was kept fixed, which is beyond the time resolution of the TCSPC setup used here. # Large χ^2 values have been encountered with the fitting of the early time decay traces, understandably due to the very weak and fast emission response. The associated error bars are indicated in Fig.2/Fig.S1.*

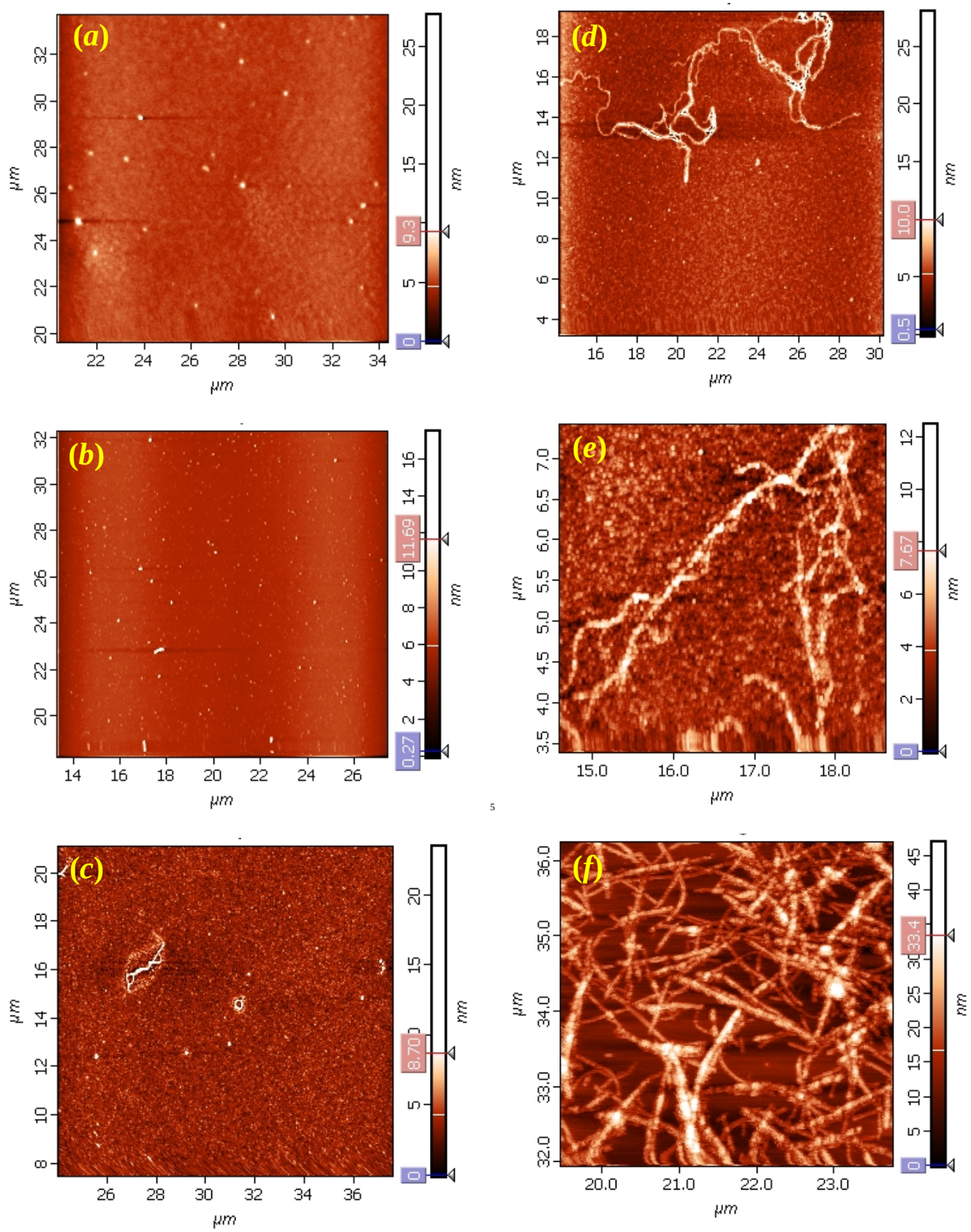

Figure S2. Figure 2 (as in main text) enlarged to view better. AFM images recorded for the insulin samples during incubation after 0 min (***a***); 65 min (***b***); 110 min (***c***); 135 min (***d***); 150 min (***e***) and 180 min (***f***).

References

(1) *The Aldrich Library of NMR Spectra*, Edition II, C. J. Pouchert, Vol. 2, p 578.
(2) V. Fodera, F. Librizzi, M. Groenning, M. Weert and M. Leone, *J. Phys. Chem. B* 2008, ***112***, 3553-3858.
(3) J. R. Lakowicz, *Principles of fluorescence spectroscopy*; Springer: New York, **2006**.
(4) O'Connor, D. V.; Phillips, D. *Time Correlated Single Photon Counting*; Academic Press: New York, **1984**.