REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

1. Name and designation of ICMR- IF : Dr. Laxman Singh Meena, PhD
   Principal Scientist

2. Address : CSIR-Institute of Genomics and Integrative Biology (CSIR-IGIB), Mall Road, Delhi-110007 (India)

3. Frontline area of research in which training/research was carried out : Protein aggregation kinetics by folding and misfolding in acidic conditions.

4. Name & address of Professor and host institute : Prof. Fabrizio Chiti, PhD
   (Full Professor)
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5. Duration of fellowship : Six Months (27/02/2017 to 26/08/2017)

6. Highlights of work conducted :

i)Technique/expertise acquired : Biophysical characterization of protein aggregation kinetics by thioflavin T binding and turbidity measurements, folding and misfolding protein in acidic conditions by different techniques like dynamic light scattering (DLS), Fluorescence spectroscopy (LS 55 spectrofluorimeter), Synergy HI Hybrid Multi Mode Reader (Gene 5).

ii) Research results, including any papers, prepared/submitted for publication : (The manuscript under preparation)

I have been work in the project entitled: Transthyretin protein aggregation kinetics: Investigation of aggregation mechanisms involved in amyloid fibril formation in disease and for their pathogenesis.

Introduction:

Transthyretin (M-TTR) is a homotetrameric protein with molecular mass of 55 kDa that synthesized only in the liver, choroid plexus of the brain, and retina of the eye [Soprano et al., 1985; Stauder et al., 1986]. we have chosen to use a monomeric M-TTR variant (M-TTR). M-TTR aggregates readily only under partially denaturing conditions, confirming that the native

monomer is not amyloidogenic and that partial unfolding of the monomer is required for amyloid formation. Although both M-TTR and tetrameric wtTTR are susceptible to aggregation under similar conditions, the rate of M-TTR aggregation is ~100 fold faster than that of wtTTR (Jiang, X., et al., 2001). Since the tertiary structural stabilities of M-TTR and wtTTR monomers are similar, this observation is consistent with rate limiting tetramer dissociation. The advantage to using M-TTR in the studies reported here is that it allows separation of the kinetics of amyloidogenesis from those of tetramer dissociation. Furthermore, the dependence of aggregation on a variety of environmental conditions (e.g., pH (3.5 to 5.5), temperature at 25^{o}C, ionic strength (7 to 160 mM), protein concentrations (0.05 to 2 mg/ml)) can be examined without having to account for the potential effect of these variables on the tetramer-monomer equilibrium.

Research Results

1. M-TTR Protein expression and purification:

To study the conformational changes during M-TTR aggregation, we expressed and purified M-TTR. First step, M-TTR purification by Anion exchanges chromatography protein purification shown in Figure: 2A and Figure 2B. 20-20 µl protein sample were collect from each selected fractions and added (4 x SDS Dye) – 10 -10 µl in each sample. Protein samples Quick centrifuge and boil in boiling water for 5-10 minutes. Centrifuge for 2-3 minutes and loaded on 20% SDS-PAGE and loaded 10-10 µl from selected protein samples shown in Figure 2B. The M-TTR purified protein resolve on SDS-PAGE as per expected size showing in the Figure.

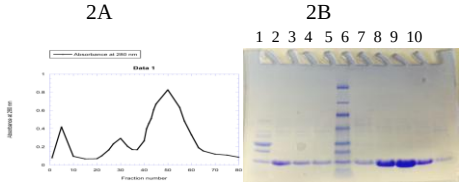

Figure 2A: M-TTR protein purification after anion exchange chromatography: Fractions number shown and taking absorbance A_{280}: Figure 2B: SDS-PAGE M-TTR protein after anion exchange chromatography: Fractions selected on the basis of absorbance taken absorbance at 280 nm: Lane1: 5; Lane 2: 30; Lane 3: 35; Lane 4: 37; Lane 5: Molecular weight Marker; Lane 6: 40; Lane 7: 45; Lane 8: 50; Lane 9: 60; Lane10: 65.

Second step, M-TTR protein purification by size chromatography shown in Figure: 3A and 3B. 20-20 µl protein sample were collect from each selected fractions and added (4 x SDS Dye) – 10 -10 µl in each sample. Protein samples Quick centrifuge and boil in boiling water for 5-10 minutes. Centrifuge for 2-3 minutes and loaded on 18% SDS-PAGE and loaded 10-10 µl from selected protein samples shown in Figure 3B.

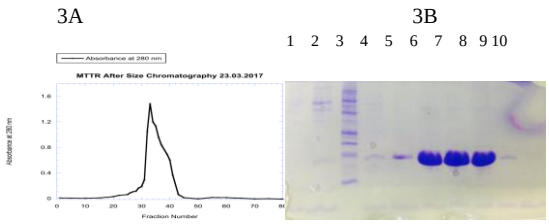

Figure 3A: M-TTR protein purification after size chromatography: Fractions number shown and taking absorbance A_{280}: Figure 3B: SDS-PAGE M-TTR protein after size chromatography: Fractions selected on the basis of absorbance taken absorbance at 280 nm: Lane1: 20; Lane 2: 24; Lane 3: molecular weight marker; Lane 4: 28; Lane 5: 30; Lane 6: 32; Lane 7: 36; Lane 8: 40; Lane 9: 45; Lane10: 50.

2. Dynamic Light Scattering (DLS) Measurements:

M-TTR proteins samples were prepared at a final protein concentration of (1 mg/ml) in 20 mM phosphate buffer, pH 7.4, 25°C. Before the measurements, the protein samples were taken all three kinds of protein samples like before centrifuge protein samples, after centrifuge protein sample and after centrifuge and filtered with anotop filters having a cutoff of 20 nm. In this study, we have observed M-TTR in monomeric form and confirmed that our M-TTR protein not aggregated at pH 7.0 and results shown in Figure 4. As an evidence that M-TTR are monomeric, as assessed the size distributions of M-TTR by means of dynamic light scattering (DLS) at pH 7.4 at 25^{o}C.

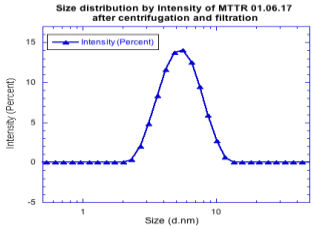

Figure 4: DLS Measurement: M-TTR protein check for monomeric form by using three different kind of protein sample like before centrifuge protein samples, after centrifuge protein sample and with centrifuge and filtered with anotop filters having a cutoff of 20 nm.

3. Fluorescence spectroscopy.

Fluorescence excitation (440 nm) and emission (560 nm) spectra were taken using a PerkinElmer LS 55 spectrofluorimeter. A plot of the ratio of the fluorescence values at two given wavelengths versus IS 120 mM concentration was obtained for various wavelengths ranging from 440/560. The 4 plots were analyzed (1). ThT alone; (2). M-TTR alone; (3). Aggregated 1 mg/ml M-TTR with IS 120 mM, pH 4.5 and (4). Aggregated 1 mg/ml M-TTR with IS 120 mM, pH 4.5 shown in Figure 5. In this experiment we concluded that M-TTR protein aggregation required acidic conditions at pH 4.5 and IS 120 mM.

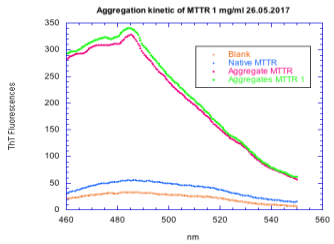

Figure 5: Fluorescence spectroscopy: Fluorescence excitation (440 nm) and emission (560 nm) spectra were using (1). ThT alone; (2). M-TTR alone; (3). Aggregated 1 mg/ml M-TTR with IS 120 mM, pH 4.5 and (4). Aggregated 1 mg/ml M-TTR with IS 120 mM, pH 4.5.

4. Aggregation kinetics of M-TTR major by Synergy HI Hybrid Multi Mode Reader: (Gene 5):

M-TTR Aggregation Kinetics, The kinetics of M-TTR aggregation mediated by acidic partial denaturation was examined by several biophysical methods. The rate and extent of aggregation are pH-dependent (pH 3.5, 4.0, 4.5, 5.0, 5.5), with maximum aggregation occurring at pH 4.5 (Jiang, X, et al., 2001), hence, all of the experiment reported herein were carried at pH 4.5, shown in Figure 6A, 6B, 6C, 6D, 6E and 6F, The rate and extent of aggregation are Ionic Strenght (IS) dependent (7 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 80 mM, 90 mM, 100 mM, 120 mM, 160 mM), with maximum aggregation occurring at IS 100 shown in Figure 7A, 7B, 7C, 7D and 7E and shown in Figure 8, hence, all of the experiment reported were carried at IS 100.

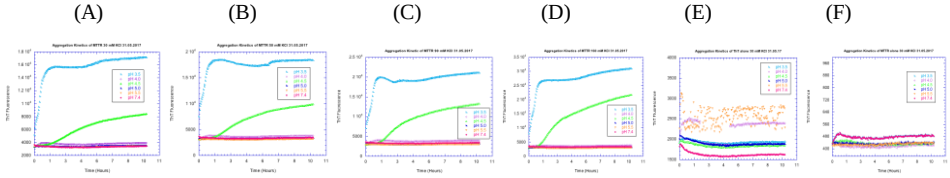

Figure 6: M-TTR aggregation kinetics with various pH like 3.5, 4.0, 4.5, 5.0 and 5.5 and various Ionic Strength like 30 mM, 50 mM, 90 mM and 160 mM: (A) Aggregation kinetics of M-TTR 30 mM KCl; (B) Aggregation kinetics of M-TTR 50 mM KCl; (C) Aggregation kinetics of M-TTR 90 mM KCl; (D) Aggregation kinetics of M-TTR 160 mM KCl; (E) Aggregation kinetics of ThT alone 30 mM KCl; and (F) Aggregation kinetics of M-TTR alone 30 mM KCl. The maximum aggregation occuring at pH 4.5.

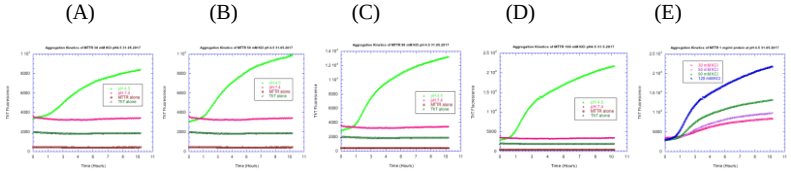

Figure 7: M-TTR aggregation kinetics of 1 mg/ml on pH 4.5, pH 7.4 and various Ionic Strength like 30 mM, 50 mM, 90 mM and 160 mM: (A) Aggregation kinetics of M-TTR 30 mM KCl; (B) Aggregation kinetics of M-TTR 50 mM KCl; (C) Aggregation kinetics of M-TTR 90 mM KCl; (D) Aggregation kinetics of M-TTR 160 mM KCl; (E) Aggregation kinetics all Ionic strength parameters (30 mM, 50 mM, 90 mM, 160 mM).

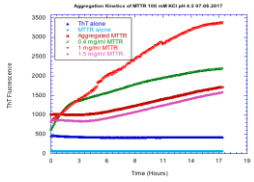

Figure 8: M-TTR aggregation kinetics on pH 4.5; Ionic Strength (IS) = 100 mM; Overnight M-TTR aggregate (1 mg/ml) protein sample as control and with various protein concentrations 0.4 mg/ml, 1 mg/ml and 1.5 mg/ml including ThT alone, M-TTR alone as a control. The maximum aggregation occuring at IS 100 mM.

Where M-TTR aggregation was measured by ThT binding shown in Figure 9A to 9F, where M-TTR proteins concentration was used at 0.2 mg/ml, 0.4 mg/ml, 1 mg/ml with aggregated

M-TTR as a positive control respectively, Again the experiment was repeat as shown in Figure 10A to 10G, IS 100 mM, pH 4.5 at 25^{o}C and results obtained maximum aggregation at 1 mg/ml.

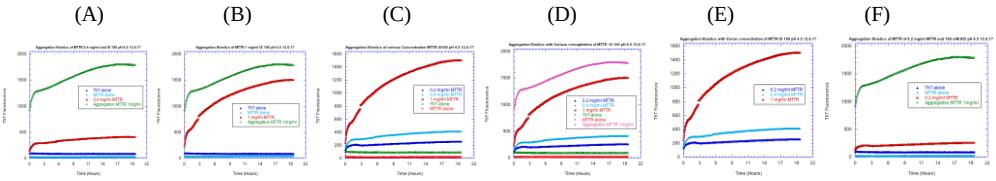

Figure 9: M-TTR aggregation kinetics on M-TTR concentration. M-TTR concentrations used 0.2 mg/ml, 0.4 mg/ml and 1 mg/ml; pH 4.5; Ionic Strength 100 mM; ThT alone; M-TTR alone as a control: (A) 0.2 mg/ml M-TTR and aggregated of M-TTR (1 mg/ml); (B) 0.4 mg/ml M-TTR and aggregated of M-TTR (1 mg/ml); (C) 1 mg/ml M-TTR and aggregated of M-TTR (1 mg/ml); (D) 0.2 mg/ml, 0.4 mg/ml, 1 mg/ml M-TTR, aggregated of M-TTR (1 mg/ml); (E) 0.2 mg/ml, 0.4 mg/ml, 1 mg/ml M-TTR with controls; and (F) 0.2 mg/ml, 0.4 mg/ml, 1 mg/ml M-TTR.

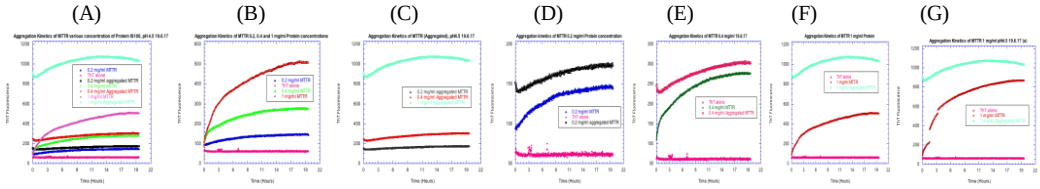

Figure 10: M-TTR aggregation kinetics on M-TTR concentrations. M-TTR concentrations 0.2 mg/ml, 0.4 mg/ml and 1 mg/ml and M-TTR aggregated 0.2 mg/ml, 0.4 mg/ml and 1 mg/ml respectively; pH 4.5; Ionic Strength 100 mM; ThT alone; M-TTR alone as a control for all parameters: (A) 0.2 mg/ml, 0.4 mg/ml and 1 mg/ml aggregation kinetics of M-TTR and aggregated M-TTR respectively; (B) 0.2 mg/ml, 0.4 mg/ml and 1 mg/ml aggregation kinetics of M-TTR along with ThT alone; (C) 0.2 mg/ml, 0.4 mg/ml and 1 mg/ml aggregated M-TTR; (D) comparasion study of 0.2 mg/ml aggregation kinetic of M-TTR and 0.2 mg/ml, aggregated of M-TTR along with ThT alone; (E) comparasion study of 0.4 mg/ml aggregation kinetic of M-TTR and 0.4 mg/ml, aggregated of M-TTR along with ThT alone; (F and G) comparasion study of 1 mg/ml aggregation kinetic of M-TTR and 1 mg/ml, aggregated of M-TTR along with ThT alone.

In Figure 11, showing M-TTR analysis of experiment data. Since the ThT signal in the presence of mature aggregates seems to vary with time, Here, we tried to normalize and calculate a normalized signal (ns) according to the following formula:

ns = [f(t)-(ThT)] / [f(a)-(ThT)]

where f(a) is the fluorescence emitted by ThT in the presence of the "mature aggregates", f(t) is the fluorescence emitted by ThT in the presence of the aggregating sample (our experiment), ThT is the fluorescence emitted by ThT in the presence of buffer only (the blank). This results showing significant aggregation occur at 1 mg/ml.

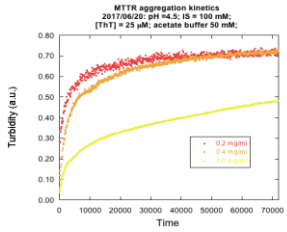

Figure 11: M-TTR aggregation kinetics; M-TTR aggregation was normalized signal (ns) ThT fluorescence contained varying concentrations of M-TTR, 0.2 mg/ml, 0.4 mg/ml and 1 mg/ml

In further study, M-TTR protein concentartion varying from 0.05, 0.1, 0.2, 0.4, 0.7, 1.0, 1.5, 2 mg/ml for aggregation as shown in Figure 12 A and 12 B and also shown in Figure 15 A and 15 B, in these experiments we analyzed that when we increase M-TTR concentration, we observed increasing M-TTR aggregation in vitro study. We further analyzed our experiment data as shown in Figure 13, as we can see, no lag phase is evident.

As per M-TTR concentrations increase, we observed more ThT fluorescence, we need to further clarification if this is real effect on M-TTR aggregation with ThT fluorescence, we further study by turbidity experiment as results shown in Figure 14 A, 14 A1, 14 B and 14 B1. We also confirm that when we increase the M-TTR protein concentration (0.05 to 2 mg/ml), we obtained increasing M-TTR aggreagation. The curve obtained by turbidity are sigmoidal in shape (Figure 14 A, 14 A1, 14 B and 14 B1) and resemble those that are frequently reported for protein aggregation at early times there is an apparent lag, followed by a phase of rapid increase in turbidity and then a plateau. The reaction is dependent on the concentration of M-TTR in three ways; At higher M-TTR concentration, (1) the maximum amplitude, or endpoint, of the turbidity is increased, (2) the length of the apparent lag phase is shortened and (3) the rate of reaction during the growth phase is accelerated. In contrast to the results obtained by turbidity, ThT fluorescence increases immediately upon mixing of M-TTR with low pH buffer (Figure 9, 10, 12 and 15). These reactions lack a lag phase and the kinetic traces can be reasonably approximately by single or double exponential fits. ThT assays are also dependent on the concentration of M-TTR; increasing the M-TTR concentration results in a faster rate and a greater extent of aggregation. The time scale of M-TTR aggregation, as measured by these two techniques is clearly different (Figure 9, 10, 12, 15 and Figure 14 for turbidity). For each M-TTR concentration examined, the reaction reaches greater than half the maximal ThT amplitude before any increase in turbidity is detected. Preliminary experiments with M-TTR suggest that it has only a minimal effect on the observed rate of reaction in this case. We chose to study M-TTR aggregation under stagnant conditions because these are easier to replicate, facilitating the comparison of data obtained by different methods. Stagnant M-TTR aggregation reactions remain homogeneous suspensions essentially until reaching completion, when the aggregates become sufficiently large to settle out of solution. This occurrence accounts for the observed decrease in turbidity (Figure 14) and the apparent increase in ThT fluorescence (Figure 12 and 15); this initial increase is followed by a decrease in fluorescence as the aggregates move through the right path. Although the data shown are truncated for clarity, reactions were allowed to proceed to completion and were then mixed prior to determination of the reaction endpoint.

(A) (B)

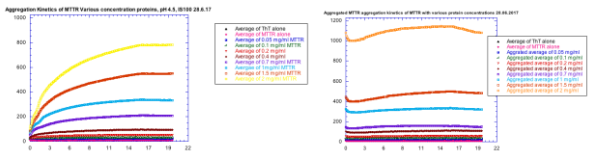

Figure 12: M-TTR aggregation kinetics on M-TTR concentration. 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml and M-TTR aggregated 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml respectively; pH 4.5; Ionic Strength 100 mM; ThT alone; M-TTR alone as a control for both experiment: (A) 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml aggregation kinetics of M-TTR, ThT alone; M-TTR alone, (B) 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml aggregation kinetics of M-TTR, ThT alone; M-TTR alone.

In Figure 13, showing analysis, here, we also find mentioned below the qpc file showing the same analysis as the above mentioned figure 11 like time [y = (signal-blank)/(aggregates-blank)], performed in this experiment with various M-TTR protein concentrations. In this experiment M-TTR analysis of experiment data. Since the ThT signal in the presence of mature aggregates seems to vary with time, Here, we tried to normalize and calculate a normalized signal (ns) according to the following formula:

$$ns = [f(t)-(ThT)] / [f(a)-(ThT)]$$

where f(a) is the fluorescence emitted by ThT in the presence of the "mature aggregates", f(t) is the fluorescence emitted by ThT in the presence of the aggregating sample (our experiment), ThT is the fluorescence emitted by ThT in the presence of buffer only (the blank). As we can see, no lag phase is evident.

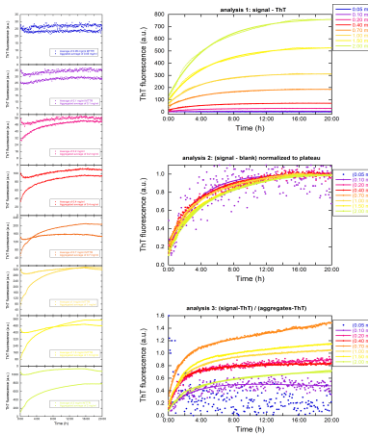

Figure 13: M-TTR aggregation kinetics; M-TTR aggregation was normalized signal (ns) ThT fluorescence contained varying concentrations of M-TTR; 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml aggregation kinetics of M-TTR, ThT alone; M-TTR alone; Upper figure show analysis 1: signal of ThT; Middle figure show analysis 2: (signal – blank) normalized to plateau; lower figure show analysis 3: (signal – ThT) /aggregates – ThT with all proteins concentration respectively.

5. Thioflavin T Versus Turbidity fluorescence Measurements:

M-TTR kinetics of aggregation measures by turbidity and ThT fluorescence are clearly different. Detailed kinetics analysis of these data is complicated, since aggregation comprises many reactions occurring simultaneously. Furthermore, the form of the rate equation describing these reactions depends on what assumptions are made concerning the mechanism of aggregation. The shape of the curve obtained by the two methods is significantly different; however, neither the turbidity nor the ThT data is well fit by the model for nucleation-dependent polymerization. Turbidity and ThT fluorescence assays were instead analyzed as follows; For each kinetic trace, the endpoint amplitude was measured, and the times required to reach 60% of the maximal amplitude was calculated. This simple yet robust method of analysis allows direct quantitative comparison of the aggregation kinetics at different protein concentrations and with different assays and provides insight into the reaction mechanism. The kinetic parameters obtained describe both the rate and the extent of reaction under a variety of conditions. Whereas the t50 for ThT reactions resembles a t1/2 because the data are nearly exponential throughout the entire reaction, the turbidity t50 is influenced both by the length of the apparent lag and the rate during the growth phase of the reaction. Values obtained at varying M-TTR concentrations for turbidity as showed in Figure 14 and ThT assays in a representative experiment (Figure 12 and 15). For each M-TTR concentration, the t50 obtained by turbidity is much longer than that calculated from ThT assays as shown in Figure 16. Furthermore, the difference between the two t50's become more pronounced at lower M-TTR concentration. Another way of comparing these data is to determine the extent of reaction measured by each of these methods (i.e., ThT or turbidity) at the time point corresponding to the t50 determined by the other method (ThT or turbidity, respectively) under the same reaction conditions. These parameters were summarized in Table 1. For each M-TTR concentration, the amount of turbidity observed is negligible (<1%) when the ThT reaction has proceeded to 60% completion. Conversely, ThT reactions are nearly complete (85-100%) by the time 60% of the maximum turbidity reaction is reached.

Table 1: Comparison of M-TTR aggregation kinetics:

M-TTR mg/ml	ThT Fluorescence			Turbidity	
	k1 26/07/2017	t ½ (ThT) 26/07/2017	k2 (ThT) 26/07/2017	t ½ (ThT) 20/07/207	In (t ½) turbidity (ThT) 20/07/2017
0.05	-	-	-	-	-
0.1	0.00010001	8.8437	6.10E-05	-	-
0.2	0.00032829	7.6551	0.00035361	27083	10.207
0.4	0.00021314	8.087	0.00020773	10833	9.2904
0.7	0.00042247	7.4029	0.00040245	5625	8.635
1	0.00051373	7.2073	0.00049346	3500	8.1605
1.5	0.00097877	6.5627	0.00090433	1613	7.3859
2	0.00064352	6.982	0.000616	2200	7.6962

Table: 1. The kinetics of aggregation of M-TTR at various concentrations are shown for a representative experiment, allowing the direct comparison of ThT fluorescence and turbidity assays. Reactions were carried out at pH 4.5 and 25^0C and t_{50} (t ½) represents the time required for the reaction to reach 50% completion,

Control reactions were carried out, to ensure that the presence of ThT in the assays does not affect the rate of aggregation. These reactions were carried out in microcentrifuge tubes in the absence of ThT. At several time points, aliquots were removed for analysis both by turbidity and by ThT. The results in these control experiments are qualitatively similar to those described above, namely that the increase in ThT precedes detectable turbidity. Furthermore, the ThT fluorescence observed at discrete time points for reactions carried out in the absence of ThT is in good agreement with that obtained in continuous ThT assays. Small differences can be seen in the amplitude of the ThT signal, attributable to some fluorescence bleaching in the continuous experiments. The rate of reaction, however, is indistinguishable, indicating that the presence of ThT does not accelerate aggregation of M-TTR. In this study, we further a comparison between ThT data and turbidity data with M-TTR aggregation kinetics normalized with formula as shown in Figure 16: $y = y\infty\ (1\text{-}e^{-kt})$ $= y\infty/2 = y\infty\ (1\text{-}e^{-kt_{½}})$ $= y\infty/2 = y\infty - y\infty\ e^{-kt_{½}}$ $y\infty/2 = y\infty\ e^{-kt_{½}}$ $= en1/2 = -kt_{½}$ $= en2 = kt_{½}$ $= t_{½} = en2/k$

This study maybe useful to determination of the nucleus size, the t_{50} was calculated for each M-TTR concentration, using either turbidity data or ThT fluorescence data. When plotted as a function of M-TTR concentration in a log-log plot, these data can be used to determine the nucleus size for aggregation.

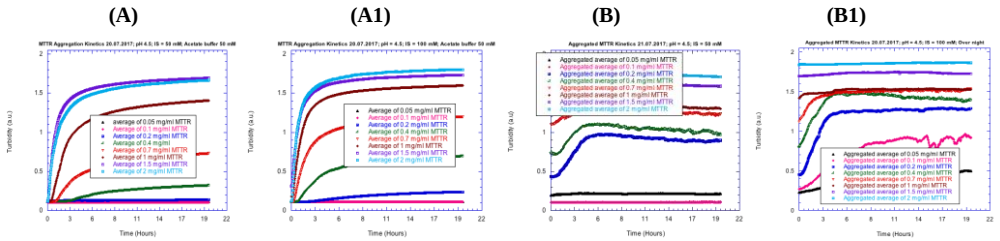

Figure 14: M-TTR aggregation kinetics on M-TTR concentration. M-TTR aggregation was measured by the increase in turbidity. Assays contained varying concentrations of M-TTR (0.05 to 2.0 mg/ml) and M-TTR aggregated 0.05 to 2.0 mg/ml respectively; pH 4.5; Ionic Strength 100 mM; (A and A1). Aggregation kinetics of M-TTR with varying concentrations 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml; (B and B1). Aggregated kinetics of M-TTR with varying concentrations 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml.

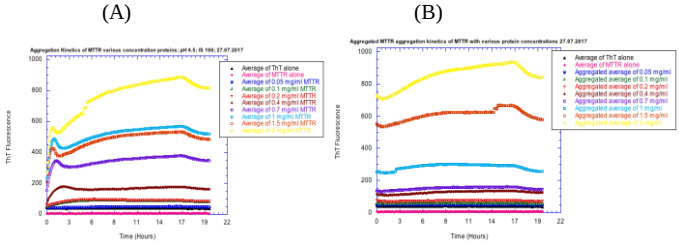

Figure 15: M-TTR aggregation kinetics on M-TTR concentration. M-TTR varying concentrations 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5

mg/ml, 2.0 mg/ml and MTTR aggregated 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml respectively; pH 4.5; Ionic Strength 100 mM; ThT alone; M-TTR alone as a control for both experiment: (A) aggregation kinetics of M-TTR, 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml, ThT alone; M-TTR alone, (B) aggregated kinetics of M-TTR 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml, 1 mg/ml, 1.5 mg/ml, 2.0 mg/ml, ThT alone; M-TTR alone,

6. Nucleus Size

The M-TTR kinetics analysis described in determination of reaction endpoints and t50s can be used to determine the nucleus size for aggregation nucleus size: As shown in figure 16, the dependence of reaction t50 values (plotted as in t50) on the concentration of M-TTR (plotted as in (M-TTR). Both the ThT data (Figure 16 A) and the turbidity data as shown in (Figure 16 B) give straight lines, the slopes of which are related to the nucleus size, n*. if aggregation is assumed to involve a preequilibrium corresponding to the formation of an oligomeric nucleus, followed by irreversible polymerization, then the slope obtained in this logarithmic plot (log-log) is equal to –n*/2 (Goldstein, R.F., and Stryer, L., 1986). For the situation where aggregation is irreversible with no preequilibrium, implying that n* = 1, the slope is equal to -1 (Goldstein, R.F., and Stryer, L., 1986). Linear regression of the data in Figure 16 A & 16 B yields a slope of -1.5 for turbidity and -1 for ThT assays. The some what steeper slope obtained by turbidity corresponds to a trimeric nucleus. A slope of -1, as obtained for the ThT data, is consistent with a dimeric nuvleus for a nucleation dependent polymerization: however, a slope of -1 is also the expectation for non nucleated process, (n* = 1; consistent with each step being both biomolecular and irreversible). In either case, the kinetics of aggregation do not show a high order dependence on the M-TTR concentration; if this is a nucleated polymerization, the nucleus size is small.

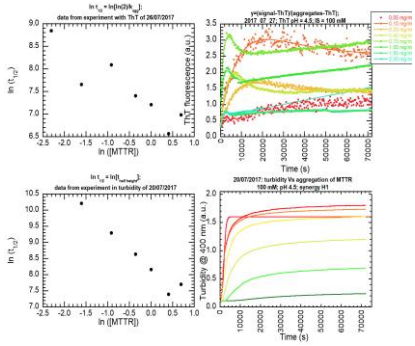

Figure 16: Determination of the nucleus size. The t_{50} was calculated for each M-TTR concentration, using either turbidity data or ThT fluorescence data. When plotted as a function of M-TTR concentration in a log-log plot, these data can be used to determine the nucleus size for aggregation.

We further comparsion study between ex-situ and in-situ data with M-TTR aggregation kinetics as shown in Figure 17.

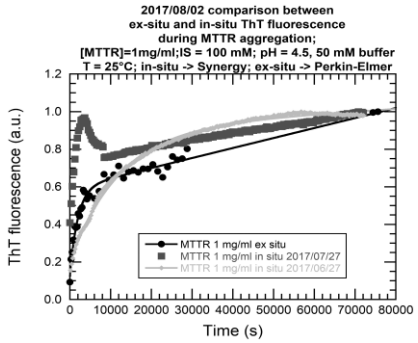

Figure 17: M-TTR aggregation kinetics, In this study a comparison between ex-situ and in-situ ThT data as collected by LS55 and synergy machine.

M-TTR aggregation kinetics, In this study a comparison between ex-situ and in-situ ThT data as collected by LS55 and synergy machine. The general trend and the time-scale do stay the same; however, the synergy still gives us this apparent two-phase behavior as shown in Figure no. 17. The study will more require understanding the mechanism of two-phase behavior.

Summary:

Transthyretin (M-TTR) is a homotetrameric protein and associated with human diseases, In our studies we reveal that M-TTR amyloidogenesis at low pH is a complex, multistep reaction whose kinetic behavior is incompatible with the expectations for a nucleation-dependent polymerization, M-TTR aggregation is not accelerated by seeding (no lag phase), and the dependence of the reaction time course is first-order on the M-TTR concentration, consistent either with a dimeric nucleus or with a non-nucleated process where each step is bimolecular and essentially irreversible. These studies suggest that amyloid formation by M-TTR under partially denaturing conditions is a downhill polymerization, in which the highest energy species is the native monomer. In this report our results emphasize the importance of therapeutic strategies that stabilize the M-TTR tetramer and may help to explain why M-TTR variants are disease-associated and also tried to understand the biological and physiological function in native M-TTR.

iii) Proposed utilization of the experience in India :

(1). To the participant ICMR-International fellowship duration, I learnt how to study protein folding and misfolding in acidic conditions, as drugs resistance for latent TB work. It happens to be a grand Challenge in Global Health. Besides, the R & D techniques used in these studies are also used in other areas of cellular & Molecular Biology. This ICMR-International fellowship would be useful in the R & D projects in which I am presently involved at IGIB.

I have been working on *M. tuberculosis* $H_{37}Rv$ to understand the mechanism of its survival/virulence with the aim to find out novel target for its intervention. This ICMR-International fellowship was a key fellowship for research and development area especially in biomedical fields. The fellowship was useful for me in many ways, including opportunity to learn the progress made in the field of biomedical sciences. This

fellowship has helped me in understanding the various approaches that are undertaken by scientific community to treat tuberculosis. In addition I have benefited from interactions with scientists from different parts of the world.

(2). While participating the ICMR-International Fellowship I took full advantage of interacting with and learning from the leading international scientists from around the world. I have brought whatever scientific literature I could get my hands on various new techniques and methodology, which I would be glad to share with anyone who would be interested in the same. I believe it will be of benefit to our institution (CSIR-IGIB) in India.

(3). Exposure of young scientists like us to international scientific community is of immense value in bringing us up-to-date with the latest research interests around the world, latest techniques being used, and in general, boosting our morale when we find that the research we are doing are of world class. Needless to say, all of the benefits of the fellowship are also benefits for our institute (CSIR-IGIB), as I am a part of the same.

(4). More fellowships should be made available for Indian Scientists in National Labs to attending ICMR-International fellowship, etc., to get scientific knowledge and share their own knowledge with the international scientific community in relevant areas. It would boost their morale as well as fill any knowledge gap in relevant areas of interest. As already mentioned in my recommendations for consideration at the India level, "As long as funds are available, support for the said papers should be automatic".

(5). Based on the results procures during this fellowship, a project proposal will be submit to funding agencies.

(6). At societal level, the R & D in the field to be very valuable if it leads to discovery of affordable, early and inexpensive diagnostic methods.

Signature of ICMR-IF
(Dr. Laxman Singh Meena)

ICMR Sanction No. INDO/FRC/452/(Y-80)/2016-17-IHD dated 24th June, 2016