Aggregation of Pmel into Functional Amyloid

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A dissertation submitted for the partial fulfillment of BS-MS dual degree in Science

Department of Chemical Sciences

Indian Institute of Science Education and Research (IISER) Mohali

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Certificate of Examination

This is to certify that the dissertation titled "**Aggregation of Pmel into Functional Amyloid**" submitted by **Priyanka Dogra** (MS09097) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Dated: April 25, 2014

Declaration

The work presented in this dissertation has been carried out by me under the supervision of Dr. Samrat Mukhopadhyay at the Department of Chemical Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bona fide record of original work done by me and all sources listed within have been detailed in the bibliography.

Priyanka Dogra
(Candidate)
Dated: April 25, 2014

In my capacity as supervisor of the candidate's thesis work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Samrat Mukhopadhyay
(Supervisor)

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Abstract:

Functional amyloids belong to a class of amyloids that are believed to have biological functions. The amyloid fibrils composed of the fragments of Pmel17 within the melanosome, acts as a template for melanin deposition. We have used the repeat domain (RPT) of this fragment for our studies as it is known to form the amyloid core which promotes melanin biogenesis. Mainly, we have focused on the aggregation studies using steady state and time-resolved fluorescence. The local environment was monitored using intrinsic tryptophan fluorescence and secondary structural changes were monitored via circular dichroism spectroscopy. The intrinsic amyloid fluorescence (blue-fluorescence) was observed during the course of aggregation which was further validated using time-resolved fluorescence spectroscopy. The nanoscale morphology of the aggregates was obtained using atomic force microscopy (AFM) and structural changes were monitored using Raman spectroscopy.

Chapter 1

1. Introduction

1.1 Basic Theory:

Amyloid fibrils are ordered protein aggregates composed of cross-β sheet structure and have been associated for a long time with human diseases like Alzheimer, Parkinson, Huntington and the transmissible spongiform encephalopathies. However, there is an emerging class of amyloids known as 'functional amyloids' that have changed the view of what amyloids have been thought in the past. These functional amyloids are found in a wide variety of organisms ranging from bacteria to human. Unlike disease associated amyloids the formation of functional amyloid is efficiently regulated by the cell as these must be put together in a dedicated way, in a directed pathway that can avert cytotoxicity and hence, results in performing physiological function that is beneficial for the organism.[1-3] Pmel17 (Premelanosome17) is a melanocyte specific transmembrane glycoprotein that arbitrates the early steps in the formation of melanosomes, highly specialized secretory lysosome related organelles of melanocytes in which melanin is synthesized and stored.[4-5] The polypeptide chain is 668 amino acid residues long and is composed of a large luminal domain containing several subdomains [Fig. 1(a)] which includes a signal sequence (SIG, 1-24 amino acid residues) which is co-transtionally removed by signal peptidase after reaching the ER lumen. An N-terminal region (NTR, 25-255 amino acid residues) which contains three highly conserved N-glycosylation sites. A polycystic kidney disease domain (PKD, 255-292 amino acid residues) is predicted to adopt a β-sheet conformation. GAP1 (292-315 amino acid residues), a repeat domain (RPT, 315-444 amino acid residues) having 10 imperfect repeats [Fig. 1(b)] each containing 13 amino acid residues that are rich in glutamic acid, proline, serine and threonine. This domain is highly modified by O-glycosylation in mature protein. GAP2 (444-515 amino acid residues), a kringle-like domain (KLD, 515-567 amino acid residues) which plays an important role in folding and secretory pathway and GAP3 (568-603 amino acid residues). A short C-terminal transmembrane domain (TM, 603-638 amino acid residues) and a cytoplasmic domain (CTD, 638-668 amino acid residues) that assists in adhering to the membrane. Further the protein is divided into two fragments, Mα (25-467

amino acid residues) and Mβ (470-668 amino acid residues).[6-7] Amyloid composed of Pmel17 is crucial in the biogenesis of melanin in mammals. The fibrils acts as a template for melanin deposition, accelerates and regulates the covalent polymerization of small reactive melanogenic precursors such as 5, 6-indolequinone (DHQ) which otherwise might diffuse across the melanosome. The release of these highly reactive and cytotoxic compounds out of the melanosome into the cytosol can have glaring effects on cellular function because these compounds acts as strong oxidizers and can react both as electrophiles and nucleophiles. It has been shown that Pmel17 forms the fibrous structure inside the melanosome. These fibrils consist of Mα fragment which gets cleaved from the Mβ fragment by proprotein convertase during translocation. Mα amyloid binding to DHQ likely reduces the entropic requirements for DHQ polymerization by increasing the effective DHQ concentration and by tailoring DHQ monomers along the Mα fiber. Hence, Mα fibrils play a critical role to set apart the reactive melanogenic intermediates thereby preventing their diffusion out of the melanosome.[1] Within this Mα fragment is the repeat domain, studies on this domain have discovered that it forms the amyloid core which promotes melanin biogenesis. RPT domain consists of a partial repeat sequence, PT.E.GTTP.QV.[6] It is known to be an IDR (intrinsically disordered region). It consists of a single tryptophan. We took the advantage of this fact in monitoring the local environment around tryptophan for elucidating the structural information of the region. So, aggregation studies were carried out in order to shed light on the environment around tryptophan during the course of aggregation which might help in further understanding of the specific regions within this domain that are prone to aggregation. While carrying out these studies we discovered that during the growth phase of aggregation reaction, apart from the peak at~ 330 nm one more peak at~ 400 nm appeared in the fluorescence emission spectrum. The intensity of the latter peak increased with aggregation time. We suspect it to be an intrinsic amyloid fluorescence as our results are in agreement with the previous reports.[8-11] The time-resolved fluorescence measurements ruled out the possible artifacts due to Raman scattering. AFM and Raman detected the presence of amyloid structures during the growth phase of aggregation. Hence, from all these results obtained from various techniques, it is possible to track the amyloid self-assembly reactions without the necessity for extrinsic fluorophores which might otherwise in some cases perturb the aggregation process. [9]

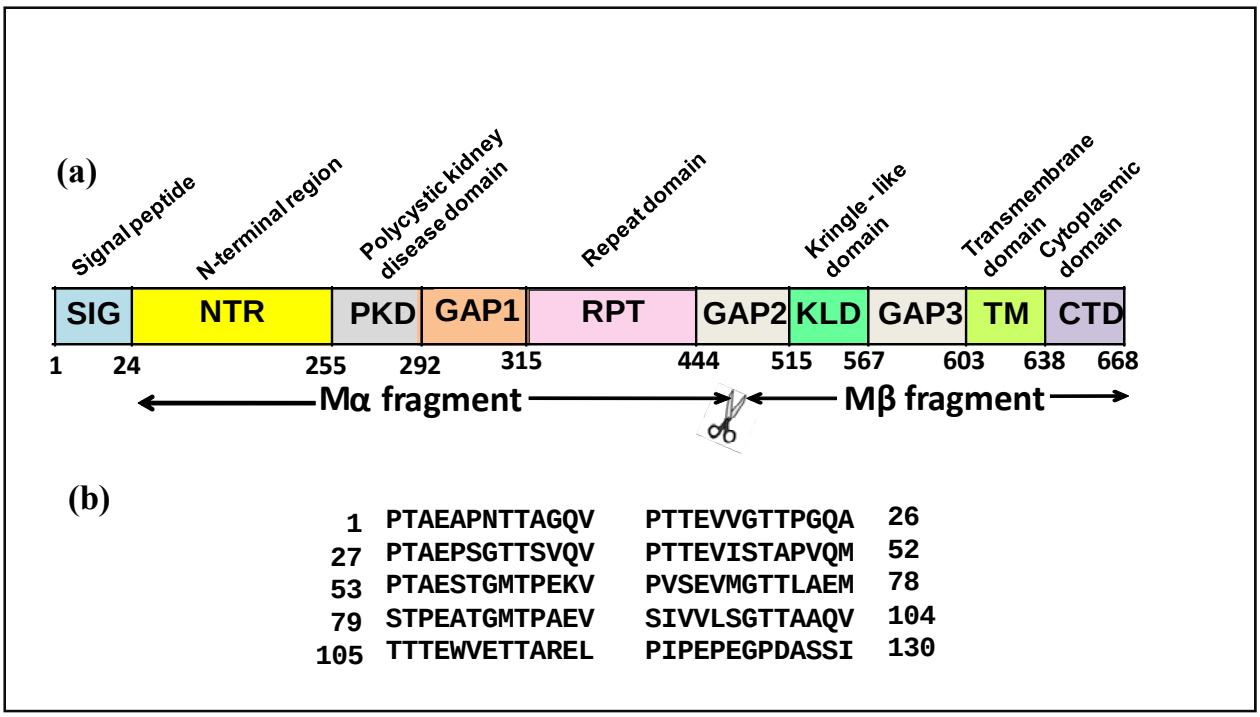


Figure 1. (a) Full-length Pmel17 is composed of 10 domains.GAP1, GAP2 and GAP3 are undefined domains. (b) Sequence of the RPT domain, showing the imperfect repeats rich in glutamic acid, proline, serine and threonine residues.

1.2 Experimental Methods:

1.2.1 Materials:

LB (Luria broth mixture powder), Tris base, glycine, SDS (sodium dodecyl sulphate) were obtained from Hi-Media. EDTA (ethylenediaminetetraaceticacid), ammonium acetate, NaOAc (sodium acetate), NaCl (sodium chloride), ThT (thioflavin T) were obtained from Sigma. Ampicillin, chloramphenicol and IPTG (isopropyl β-D-1-thiogalactopyranoside) were purchased from Goldbio.Com. HCl (hydrochloric acid), ethanol, glacial acetic acid and ammonium sulphate were purchased from Merck. Q (quaternary ammonium) sepharose fast flow resin was purchased from GE Healthcare. Superdex G-75 resin was obtained from Sigma and streptomycin sulphate was obtained from CDH and used as received.

1.2.2 Protein Expression and Purification:

The pET 21a (+) plasmid with RPT domain gene was a kind gift from Prof. Jennifer C. Lee from National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, USA. It was transformed into BL21 (DE3) pLysS strain of *Escherichia coli*. Briefly, 1 % of the overnight grown culture (containing 100 μg/mL ampicillin and 35 μg/mL chloramphenicol) of BL21 (DE3) pLysS was transferred into fresh media (containing 100 μg/mL ampicillin) and when the OD at 600 nm reached to 0.6-0.7, the cells were induced with 100μM IPTG for 4 hours. To obtain the cell pellet, culture was centrifuged at 4,000 rpm for 30 min at 10°C. Pellet was resuspended in lysis buffer (10 mM Tris, 1mM EDTA, pH 7.5) and was stored at -80 °C until further use. Pmel repeat (RPT) domain was purified using ammonium sulphate precipitation method followed by anion exchange and size exclusion chromatography. Ammonium sulphate precipitation method was done by boiling the lysed cells at 95°C for 30 min followed by centrifugation at 11,500 rpm for 30 min at 10 °C. The supernatant was collected and thoroughly mixed with 136 μL/mL of 10 % streptomycin sulphate and 228 μL/mL of glacial acetic acid followed by centrifugation at 115000 rpm for 30 min at 10 °C. To the clear supernatant, equal volume of saturated ammonium sulphate was added and kept at 4 °C with an intermittent mixing for 1 hour. The precipitated protein was separated by centrifugation at 115000 rpm for 30 min at 10°C was suspended in 1:5 ratios of 100 mM ammonium acetate and ethanol, centrifuged at 4,500 rpm for 10 min at 10 °C. Finally, the

pellet obtained was washed with absolute ethanol (chilled) and was air dried. The dried pellet was suspended in 10 mM Tris, pH 7.5 and further purified by FPLC (fast performance liquid chromatography) on a Q Sepharose column and the protein was eluted at ~ 50mM NaCl.[12] The fractions containing protein of interest was concentrated and was further purified by FPLC on a SuperdexG-75 column. The purity of the collected fractions was appraised by SDS-PAGE [Fig. 2].

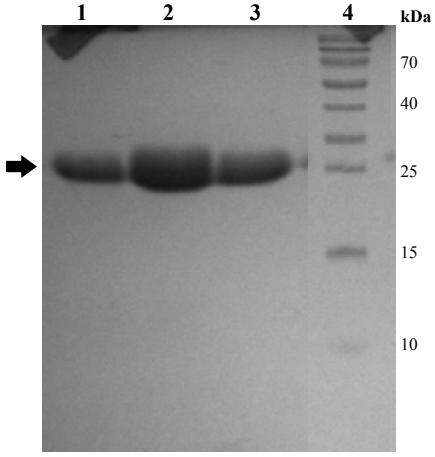


Figure 2. SDS-PAGE (15%) analysis of the RPT domain, the arrow shows pure fractions. Lane 1-3 corresponds to the protein fractions and lane 4 corresponds to the molecular weight marker.

1.2.3 Pmel17 RPT domain aggregation:

Prior to every experiment, the RPT domain of Pmel 17 was denatured using 8 M urea, 10mM Tris pH 7.8 for overnight at 4°C.The protein in denaturant was then exchanged with pH 5.0 buffer (50 mM NaOAc, 100mMNaCl) using PD-10 Desalting column and further concentrated by 3kDa molecular weight cut-off (MWCO) Microcon filter (Millipore).The concentration of the RPT domain was determined by measuring tryptophan absorbance usingε_{280}=5,690 $M^{-1}cm^{-1}$(Scripps protein calculator v3.3).[13] The measurements were carried out using 1cm pathlength cuvette. The final concentration of protein was 35μM in the aggregation buffer 50 mM NaOAc, 100mM NaCl pH 5.0.The reaction was set up on a

magnetic stirrer at 37°C and 600 rpm and ThT was used as an extrinsic fluorophore for monitoring the aggregation reaction. [14]

1.2.4 Circular Dichroism (CD) experiments:

Far UV-CD spectra were collected on Chirascan CD spectrometer (Applied Photophysics, UK) using a 1 mm path length quartz cuvette at 25°C. The concentration of RPT domain of Pmel17 was 20μM for all CD experiments. All the spectra were recorded in the range of 205-260 nm, with a scan rate of 0.5 nm/s and averaged over three scans. The final spectra were buffer subtracted and smoothened using Chirascan 'ProData viewer' software provided with the instrument.

1.2.5 Steady state-fluorescence measurements:

All steady state experiments were carried out on Fluoromax-4 (Horiba Jobin Yvon, NJ) using 10 mm x 1 mm quartz cuvette at 25°C. For tryptophan fluorescence experiments, the spectra were collected using the following parameters: λ_{ex} = 295, 305 nm, λ_{em} (range) = 310-500 nm, excitation bandpass = 1 nm and emission bandpass = 5.5 nm. For ThT fluorescence experiments, the spectra were collected using the following parameters: λ_{ex} = 400 nm, λ_{em} (range) = 460-590 nm, excitation bandpass = 2 nm, emission bandpass = 2 nm. All the spectra were recorded with an integration time of 0.5 seconds and averaged over at least three scans. The spectra were extrapolated using OriginPro Version 8.5 software.

1.2.6 Atomic force microscopy:

AFM images were acquired using a MultiView 2000^{TM} instrument (Nanonics Imaging Ltd., Jerusalem, Israel). The instrument was operated in an intermittent mode and normal force tuning-fork, phase feedback technique. For fibril imaging, Cr-coated cantilevered glass probe of diameter ~10 nm oscillating at a resonance frequency of 32.01 kHz was used. Images were collected in sample scanning configuration on Quartz software (provided with the MultiView) in a scan area of 2 x 2 μm at a resolution of 250-400 pixels. Before collecting the AFM images, the aggregation buffer (50 mM NaOAc, 100 mM NaCl) was filtered using 0.22 μm membrane filter (Millipore). 10μL of fibrillar sample was deposited onto the freshly

cleaved surface buffer-washed muscovite mica (Grade V-4 mica from SPI, PA) and air dried for 15 minutes and then was rinsed with 20 µL of aggregation buffer pH 5.0. The sample was left to dry completely under gentle stream of nitrogen gas for 20-30 minutes before collecting the images. Then an area of interest was chosen and the zoomed area was scanned further for collecting the images. The images were processed and analyzed using WSxM version 4 develop 11.6 software available with the AFM set-up The height profiles were also produced using the WSxM software to obtain topographic information about the protein aggregates.[15-16]

1.2.7 Raman Spectroscopy:

The Raman spectra of the Pmel RPT domain aggregates were collected on an inVia Raman microscope (Renishaw, UK) at ~25 ºC. The fibrils were precipitated using acetone (1:10). The samples were then kept at -20°C for one hour and then centrifuged at 115000 rpm for 10 minutes at 10°C. Pellets obtained were once again washed with acetone and were deposited on the slide containing aluminum foil which was rinsed with ethanol. A laser line of 785 nm (350mW) from a HPNIR laser was selected as an excitation source that was focused into the sample by a 20X objective (Nikon, Japan).All the data were baseline corrected using cubic spline interpolation method (available in the Wire 3.1 software).

1.2.8 Time-resolved fluorescence measurements:

The time-resolved fluorescence decays of the samples were collected using a time correlated single-photon-counting (TCSPC) setup (Fluorocube, Horiba Jobin Yvon, NJ). 295 nm and 340 nm LED (Light Emitting Diode) was used as excitation source having repetition rate of 1 MHz. All the decays were collected at magic angle (54.7°) with 12 nm bandpass and a PhotoMultiplier Tube (PMT) (Hamamatsu Corp) was used as detector. An aqueous solution of 2 % ludox was used to collect the instrument response function (~1.1 ns). In order to obtain a good signal to noise ratio, 10,000 counts were collected at the peak. All the experiments were carried out at 25°C.

Chapter 2

2. Summary and Conclusions

2.1 Results and discussion:

2.1.1 Circular Dichroism measurements to shed light on the secondary structural elements present in the RPT domain of Pmel17:

The repeat domain was purified under the native conditions. After purification, circular dichroism (CD) experiment was performed for the RPT domain. We observed that the spectrum showed two minima, one at 222 nm and another at 208 nm, which is a characteristic feature of α- helical structure but according to the reports it is an intrinsically disordered region. [6, 14, 17] Fig. 3 shows the CD spectrum of the RPT domain is in α-helical state. This discrepancy could be explained by the possibility of incorporation of some other amino acids in the repeat sequence (see later).

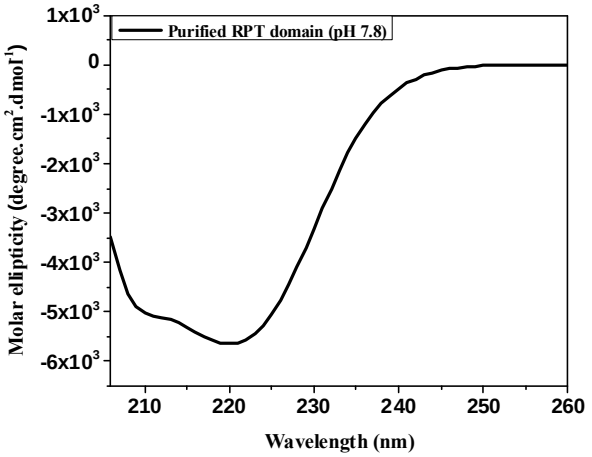


Figure 3. CD spectrum of the RPT domain when purified under native conditions showed α-helical structure.

2.1.2 Aggregation studies for the RPT domain:

The aggregation reaction was monitored using two probes, fluorescence intensity and CD. For the aggregation studies, amyloid marker Thioflavin T (ThT) was used to monitor fibril formation. Enhancement in the ThT fluorescence intensity was seen during the course of aggregation reaction. Fig. 4(a) shows the binding of ThT (20μM) to RPT fibrils, affirming the formation of amyloid structure. Using CD measurements, the initial structure of the protein in aggregation buffer pH 5.0 was found out to be α-helical. When the aggregation reaction reached saturation, the CD spectrum was collected. A change in the conformation was observed from α-helical to a partial β-sheet .According to one of the reports by Lee and co-workers, the RPT fibril tends to dissolve at pH >6 i.e. the protein goes back to its native conformation.[6, 14] We have also observed this reversible conformational change from the partial β sheet form to highly α-helical form by dissolving the fibrils into Tris buffer pH 7.9. Fig. 4(b) shows above mentioned conformational changes.

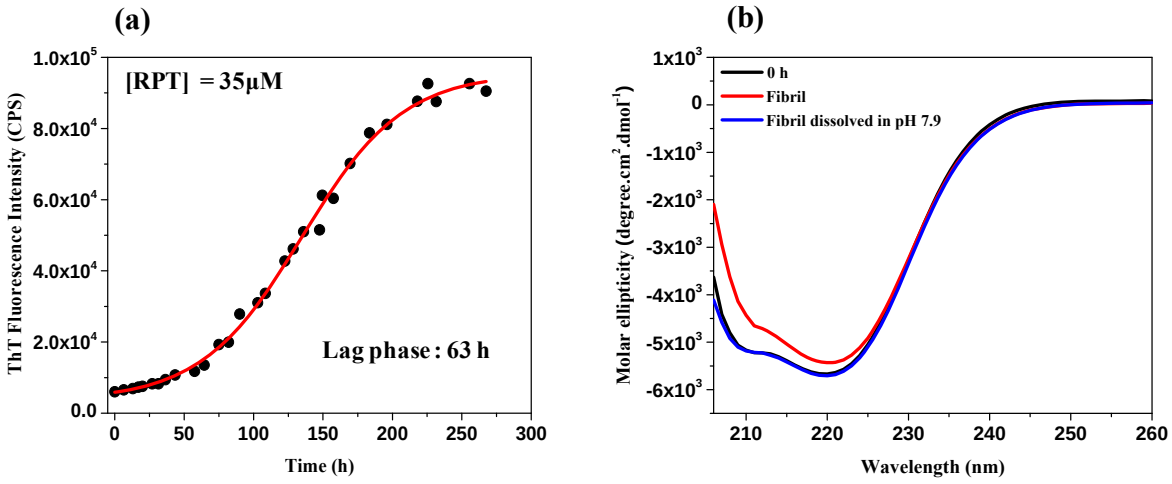
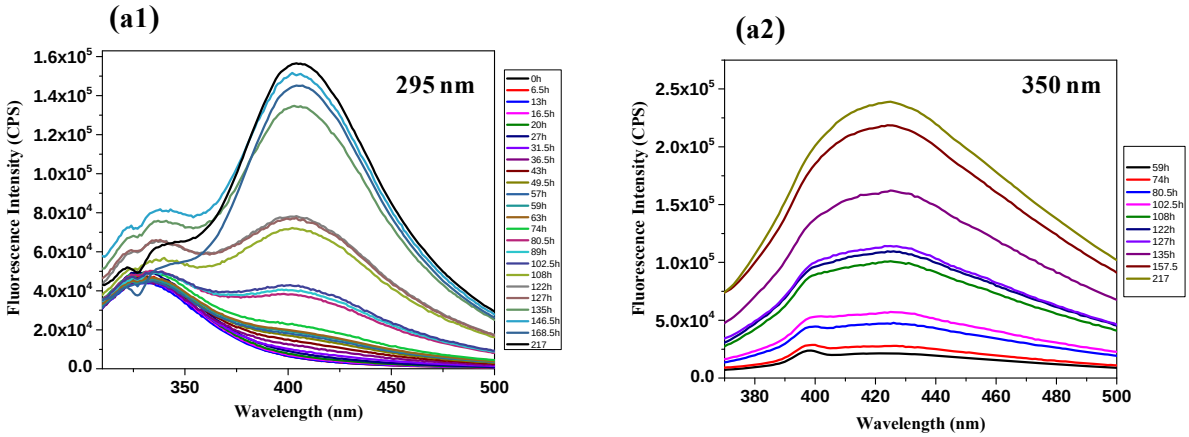


Figure 4. (a) RPT fibrils monitored by thioflavin T over time at pH 5.0. (b) Circular Dichroism spectra of soluble and fibrous RPT.

2.1.3 Intrinsic amyloid fluorescence signature during Pmel RPT domain aggregation:

The motive behind studying the intrinsic fluorescence was to shed light on the local environment around tryptophan during the course of aggregation so that the structural information obtained from the studies can further help in understanding that which all

regions within the polypeptide chain are prone to aggregation. While carrying out the experiments we observed the intrinsic fluorescence in the visible range i.e. a peak at~401 nm, a serendipitous discovery [Fig. 5(a1) and (a2)].[8] When seen under UV-light, blue fluorescence was observed [Fig. 5(b)]. There could be various possible reasons behind this intrinsic blue fluorescence. First, as aggregates are composed of the cross-β sheet structures which is formed via wide ranges of hydrogen bonds, there might be sufficient delocalization of the electrons associated with the formation of hydrogen bonds that results into a low energy transition while emission.[9] Second, as the peptide bond has a double bond character due to the resonance of electrons between the C=O and C-N bonds, it might have happened that these very electrons were further delocalized due to the engagement of peptide bond in hydrogen bonding during the process of aggregation.[10] Third, it could be due to the energy transfer between the aromatic amino acid residues i.e. tryptophan (W), tyrosine(Y) and phenylalanine (F). As there is no Y and F in the RPT domain, therefore, the only possibility that exists is the energy transfer between the tryptophan's. Tryptophan emission peak is usually observed at ~345 nm. Since we are observing emission maxima at ~ 401 nm [11], we speculate that it could be due to some kind of energy transfer which can also lead to the formation of the excimer. Fourth, there could be an energy transfer from an aromatic residue to peptide bond or vice-versa.



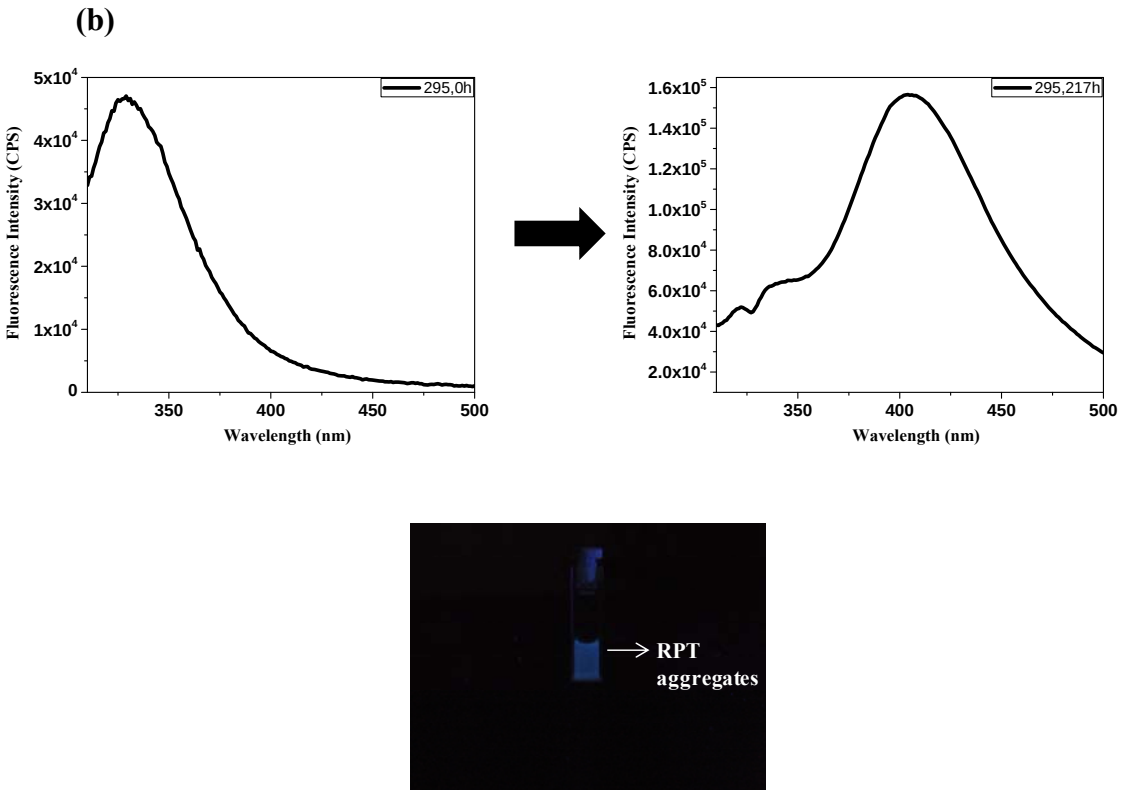


Figure 5. (a) Fluorescence intensity observed in the visible region, (1) when excited at 295 nm (2) when excited at 350 nm. (b) RPT domain during 0h (left) and 217 h (right) of aggregation process (excitation at 295 nm) showed blue fluorescence when seen under UV light.

2.1.4 Intrinsic amyloid fluorescence measurements allow the direct traverse of amyloid self-assembly:

The intrinsic amyloid fluorescence has the ability to envision the protein amyloid structure without any kind of extrinsic labeling. ThT and Congo red are the two most widely used extrinsic fluorophore for tracking the aggregation reaction because their fluorescence property alters upon binding to amyloid structures. In spite of the fact that these extrinsic fluorophores have so many applications, the use of such probes in some cases can perturb the aggregation process. For example, some compounds have been erroneously discovered as aggregation inhibitors because of reduced amyloid binding affinity due to direct interaction with ThT. On the other hand, the intrinsic fluorescence does not require binding of external

probes or any chemical modification that might otherwise can quench the fluorescence resulting into a faulty outcome. Figure 6 shown below enables to monitor the aggregation reaction free from the use of any external fluorophore. Hence in certain cases without the use of any external probe the amyloid self-assembly can be traced by taking the advantage of intrinsic amyloid fluorescence.[9]

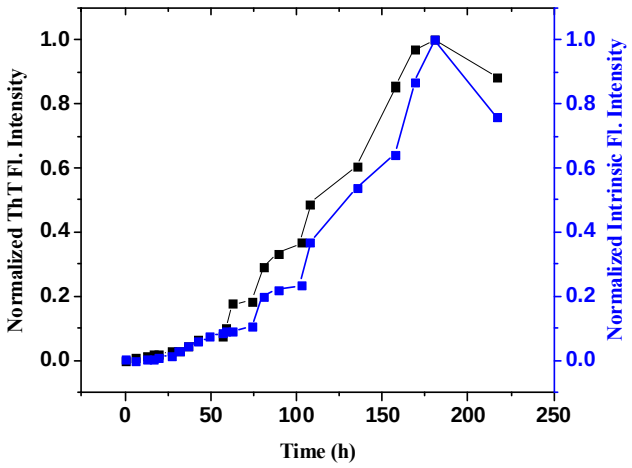


Figure 6. Comparison of intrinsic fluorescence and ThT assays for RPT domain.

2.1.5 Time-resolved fluorescence measurements:

Analysis of the time-resolved intensity confirmed that the peak that appeared at~401 nm is indeed due to fluorescence. When the sample containing the RPT fibrils was excited at 295 nm and emission was seen at 340 nm as well as 401 nm, the lifetime analysis showed the presence of short lifetime for 340 nm as compared to 401 nm indicating the presence of some kind of energy transfer or electron delocalization occurring in the excited state [Fig. 7 (a)]. Further the sample was excited using 340 nm LED and emission was collected at 401 nm. The longer lifetime component of tryptophan was 6.60 ns which is much longer than the usual lifetime component of tryptophan [Fig. 7(b) and Fig. 7(c)]. Hence we speculate that the longer lifetime component can be due to some kind of energy transfer between the tryptophan's leading to the formation of tryptophan excimer and/or intrinsic blue

fluorescence that has been seen earlier for protein aggregates.[8-11] The fluorescence lifetimes data are provided in Table 1.

Table 1.

Excitation wavelength	Emission wavelength	Fluorescence lifetime
295 nm	340 nm 401 nm	τ_1 = 1.12 ns ; τ_2 = 4.95 ns ; τ_{avg} = 1.86 ns τ_1 = 2.12 ns ; τ_2 = 6.12 ns ; τ_{avg} = 3.54 ns
340 nm	401 nm	τ_1 = 1.44 ns ; τ_2 = 6.60 ns ; τ_{avg} = 2.94 ns

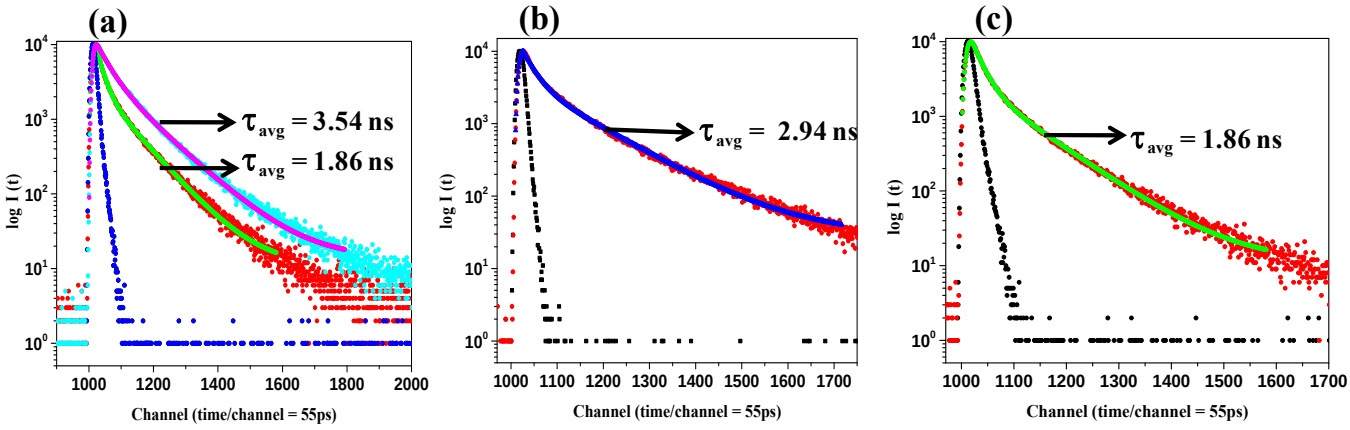


Figure 7. (a) The time-resolved fluorescence intensity decay showed longer lifetime component for the sample emitted at 401 nm as compared to 340 nm (excitation at 295 nm).(b) The time-resolved fluorescence intensity decay (excitation at 340 nm and emission at 401 nm).(c) The time-resolved fluorescence intensity decay (excitation at 295 nm and emission at 340 nm).

2.1.6 Raman spectroscopy and AFM microscopy characterize the protein's amyloid state:

In order to delineate the arrangement of the cross-β sheets in the amyloid state Raman spectroscopy was performed. Following signatures were obtained from the Raman spectra of RPT fibrils [Fig. 8(a)]. The peaks at 621 cm^{-1},1033 $cm^{-1,}$ and 1607 cm^{-1}corresponds to phenylalanine. A sharp peak at 1004 cm^{-1} corresponds to phenylalanine asymmetric breathing. Peaks at 645 $cm^{-1,}$ 1174 cm^{-1} and 1208 cm^{-1} corresponds to tyrosine. Amide IIIβ [β (antiparallel β-sheets) + Random coil] band was observed at 1239 cm^{-1}. A band at1406 cm^{-1} showed the presence of C_α-H fibril core β-sheet. A peak at 1345 cm^{-1} showed tryptophan C_α-H deformation while the peak at 1456 cm^{-1} showed CH in-plane deformation. A sharp intense peak at 1670 cm^{-1} showed the presence of amide I (cross-β-sheet/Polyproline-II conformation) in the fibrils.[16] Next, the morphology of these fibrils was investigated using AFM. From the AFM images we were not able to observe the exact morphology of the fibrils. Heterogeneous populations of the aggregates were observed. The average height for the first set of population ranges from 6-10 nm [Fig. 8(b1), 8(b2)] and for the second set of population it ranges from 20-32 nm [Fig. 8(c1), (c2)].[15]

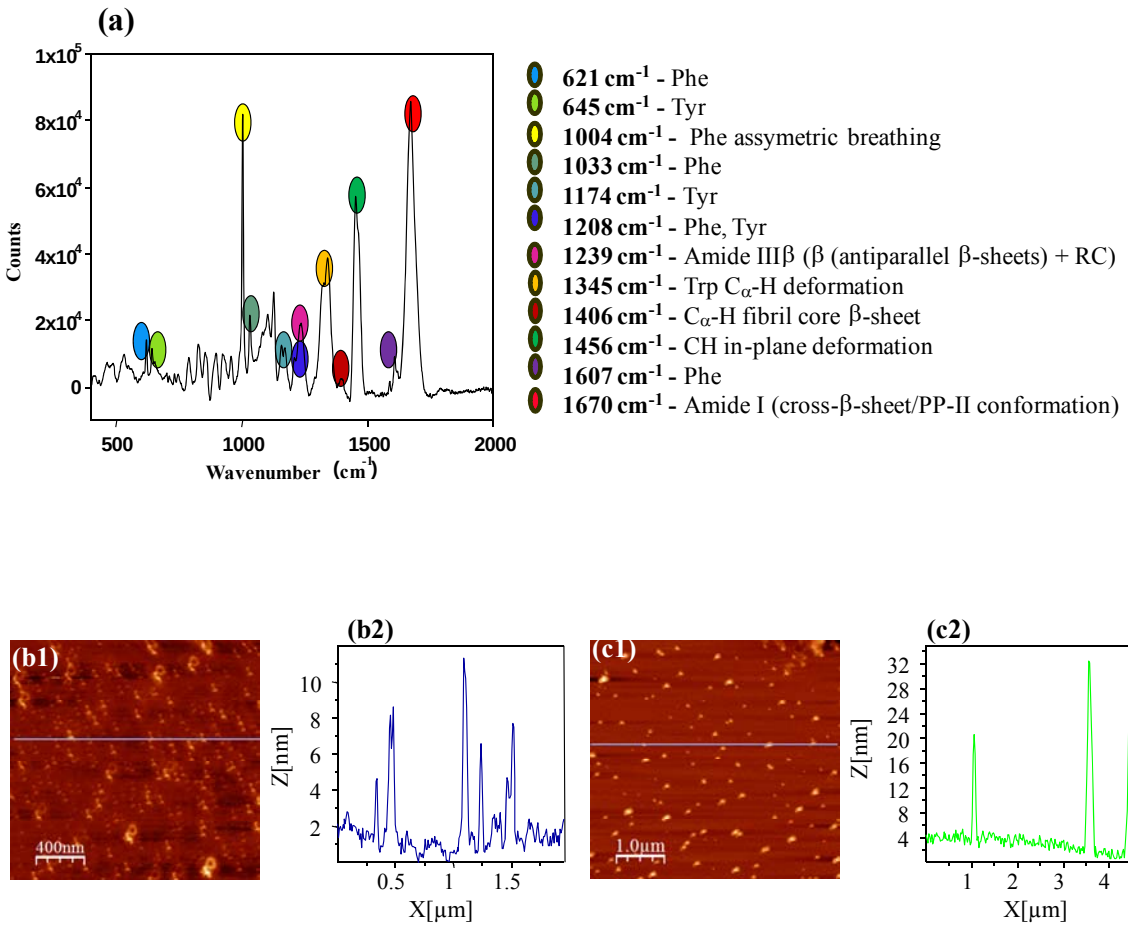


Figure 8. (a) Raman spectra of the fibrils of RPT domain showed that the fibrils are rich in phenylalanine and tyrosine. (b1) AFM image of the fibrils (first set of population) at 25°C, (b2) Height profile of the fibrils (first set of population) at 25°C. (c1) AFM image of the fibrils (second set of population) at 25°C, (c2) Height profile of the fibrils (second set of population) at 25°C.

2.2 Conclusion and Future Prospects:

We were able to obtain preliminary results on the aggregation of RPT domain. However, some results obtained from our studies were not corroborating the previous observations on the RPT domain of Pmel 17. This domain is reported to be an intrinsically disordered region with a random coil conformation but our results showed it to be highly α-helical. The CD for the fibrils should demonstrate predominant β-sheet structure, but in our case still some α-helical content was predominant. According to the reports the fibrils once formed immediately dissolves in pH > 6 but we were able to observe this after five days. There is no phenylalanine (Phe) and tyrosine (Tyr) in this domain but Raman data showed the presence of both of these amino acids. AFM images showed completely different morphology (heterogeneous populations with average height varying from 6-10 nm to 20-32 nm) of the RPT fibrils and no long straight fibrils were seen which is mentioned in the reports. It is possible that there were incorporations of Phe and Tyr in the sequence since we have used BL21 (DE3) pLysS bacterial strain that can result in the codon biasing. Therefore, the future aim will be to transform the RPT domain plasmid into BL21 (DE3) RIPL bacterial strain to obtain the right sequence. Additionally, we will also carry out mass spectrometry and amino acid sequence analysis. We are also interested in examining which region of the RPT domain is more prone for aggregation, we will also examine whether fibrils of other proteins are promoting melanin synthesis to the same extent as the RPT domain does. In addition to that we are interested in elucidating the role of different domains of Mα fragment in melanin biogenesis. Finally we will try to decipher the mutations responsible for skin disorders (e.g. Albinism) and to create those mutations, which will help in monitoring its effect on the deposition of melanin.

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