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Diameter of the vial plays a crucial role in the amyloid fibril formation: Role of interface area between hydrophilic-hydrophobic surfaces

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ABSTRACT

Number of incurable diseases associated with neurodegenerative syndromes like Alzheimer's, and Parkinson's, are owing to protein aggregation which leads to amyloid fibril formation. In vitro, such fibrillation depends on concentration, temperature, pH, ionic strength, organic solvents, agitation, and stirring, which play a crucial role in understanding the mechanism of fibrillation as well as to identify potential inhibitors for fibrillation. Although these parameters were considered, the precise repeatability of amyloid fibrillation kinetics between laboratories remains challenging. Herein, we have demonstrated that another important parameter such as diameter of the vial in which protein undergoes fibrillation play a key role in the amyloid fibrillation. The various biophysical analyses indicated that the lag time, elongation, and the amount of fibril formation was significantly reduced with decreasing the diameter of the reaction vial from 24 to 15 mm. Further, the minimum amount of protein required for fibrillation was determined by the diameter of the vial. The observed fibrillation difference in different vials is most likely due to the variation in the interface area between hydrophobic (air) and hydrophilic (water) surfaces as the diameter of the vial changes. The current results have a major role in the design of drug screening assays for amyloid inhibition.

1. Introduction

Under appropriate conditions, proteins and peptides undergo self-aggregation that leads to the formation of amyloid fibril, which was found to be responsible for a number of diseases associated with neurodegenerative syndromes like Alzheimer's, Parkinson's, Prion, *etc* [1–4]. These amyloid fibrils are rigid, self-assembled, fibrillar, and β-sheet rich in structure [5,6]. The conversion of secondary structure from α-helix/unordered to β-sheet is believed to be the early step in the onset of amyloid fibrillation which is driven by intermolecular hydrophobic interactions [6]. Although the exact molecular mechanism of amyloid fibrillation is remained elusive or under debate, it is well demonstrated that, *in vitro*, the fibrillation process follows a cooperative transition that consists of three phase (sigmoidal type) namely lag phase (nucleation), elongation (growth) followed by saturation [7]. The nature of sigmoidal curve is not only important to understand the molecular mechanism of amyloid fibrillation but also important in finding amyloid inhibitors (natural and synthetic molecules) *in vitro* [8–12]. In the latter case, the efficiency of inhibitor was determined based on how far the inhibitor increase the lag time as well as decrease the amount of fibrillation (magnitude of saturation). In order to find whether the selected molecule is inhibiting amyloid fibrillation, one has to select an *in vitro* condition at which protein undergoes fibrillation (control experiment) and also should maintain the "identical" experimental parameters for the protein in the presence of inhibitor(s) as amyloid fibrillation is highly sensitive process. The reported parameters include concentration, temperature, low pH, ionic strength, organic solvents, agitation, and stirring [13–18], which play a crucial role to trigger or accelerate the fibril formation. Although these parameters are mentioned in the literature report, the reproducibility of amyloid fibrillation kinetics (lag phase and magnitude of saturation) remains challenging. While pre-solvent (1,1,1,3,3,3-Hexafluoroisopropanol, HFIP or 2,2,2-Trifluoroethanol (TFE) containing 0.01–0.1% Trifluoroacetic acid) treatment method [19,20] provides repeated results in a laboratory, the reproducibility between different laboratories is still remain inconsistent though the experimental parameters mentioned in the literature were kept identical. Meanwhile, we have recently shown that peptides can form self-assembly when dried from HFIP and TFE solutions [21,22]. So far, the origin for the differ-

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ence in the amyloid kinetics is not clearly identified. In this context, during our amyloid fibrillation studies, we perceived that vials with different diameters showed significant difference in the kinetics as well as quantity of amyloid fibrillation although other above said parameters are identical. This preliminary observation encouraged us to explore the importance of the diameter (spatial confinement) of the vial on the kinetics of amyloid fibrillation which in turn has significant impact on the search of amyloid inhibitors during drug discovery. The outcome of this study is thus not only providing the reason for the lack of reproducibility in the amyloid kinetics but also provide an additional knowledge on the role of interface area between hydrophilic (water or liquid) and hydrophobic (air) surfaces on the amyloid fibrillation.

2. Materials and methods

2.1. Materials

Insulin (I5500), Lysozyme (L6876), κ-casein (C0406), and ThT (T3516) were purchased from Sigma Aldrich (USA). Acetic acid was purchased from SRL chemicals, India. 35% Hydrochloric acid (HCl), Toluene (General Reagent grade) was obtained from Merck Pvt. Ltd (Mumbai, India). Ethanol was purchased from s d fine chemicals limited, Mumbai, India.

2.2. Insulin fibril formation

Insulin fibrillation was induced by following the previously reported method [23]. Briefly, a stock solution of insulin (2 mg/mL) was prepared in 20% v/v acetic acid (AcOH) solution containing 100 mM NaCl (pH 2). This stock solution was diluted to get the final concentration of insulin (0.1–0.2 mg/mL) and 3 mL of total volume of solution. This solution was equally transferred into three vials with diameter of 15, 20 and 24 mm. The fibril formation was monitored simultaneously by incubating the insulin solutions at 65 °C without stirring for a period of 300 min.

2.3. Lysozyme fibril formation

Lysozyme fibrillation was induced by following the previously reported method with minor modification [24]. A stock solution of lysozyme (6.25 mg/mL) was prepared in water (pH 2, adjusted using HCl) containing 100 mM NaCl and 10% v/v ethanol. The stock solution was diluted to get the required concentrations of lysozyme (1.25 mg/mL). The fibril formation in the presence of air-water interface and in the absence of air-water interface where air was replaced by toluene was monitored by incubating the lysozyme solutions at 65 °C with stirring 150 rpm for a period of 240 min. In the case of water-toluene interface, 4 mL of toluene was added on the top of water. Fibril formation in different diameter vials was studied simultaneously.

2.4. Seeding experiments

Seeding experiments were performed by following the method which is commonly used in protein fibrillation [25]. Briefly, 50 μL of preformed lysozyme fibrils (formed in 24 mm diameter vial) were added into the each vials (15, 20, and 24 mm) which contain lysozyme at a concentration of 1.25 mg/mL. All samples were incubated at 37 °C without stirring for over a period of time.

2.5. κ-Casein fibril formation

κ-Casein fibrillation was induced by following the previously reported method [26]. Briefly, a stock solution of κ-casein (2 mg/mL) was prepared using 50 mM sodium phosphate buffer containing 100 mM NaCl (pH 7.2). Appropriate volumes of these stock solutions were diluted using the same buffer into different diameter of the vials. The final concentration of κ-casein was 0.125 mg/mL. Fibril formation of κ-casein was assessed by incubating the samples present in different diameter of the vials at 37 °C, without stirring, for ~2 days.

2.6. Turbidity measurements

The turbidity change associated with fibrillation of insulin, lysozyme and κ-casein was monitored by measuring the absorbance change at 600 nm [27] for insulin and κ-casein (A_{600}) while 450 nm [24] for lysozyme. At different time intervals, the incubated samples were transferred to a quartz cell (1 cm) for absorbance measurement. All absorbance was measured using a Perkin Elmer (Lambda EZ 201) Ultra Violet–Visible (UV/Vis) spectrophotometer at 25 °C. All experiments were performed in triplicate and error bar in the corresponding figure represents the overall output of the three individual experiments except κ-casein fibril formation.

2.7. Thioflavin T (ThT) fluorescence measurements

All fluorescence spectra were recorded using a Carry (eclipse) fluorescence spectrometer at 25 °C. A fresh stock solution of ThT was prepared in 10 mM phosphate buffer containing 150 mM NaCl (pH 7). At the time of measurements, a constant volume (20 μL) of insulin (different vials) and lysozyme (different vials) amyloid fibrils was added to 2 mL of ThT solution. The final concentration of ThT was 50 μM. Before fluorescence measurement, the solution of sample mixtures were shaken well as the intensity of ThT fluorescence depends on the amount of fibrils added. The fluorescence spectra were measured from 460 to 660 nm with a scan rate 100 nm min^{-1}. A quartz cell with a path length of 1 cm was used. The excitation wavelength was 440 nm.

2.8. Atomic force microscopy (AFM) measurements

AFM images were taken using NT MDT (NTEGRA PRIMA) Instrument (Russia). The resonant frequency and force constant was set at around 250 kHz and around 13 N/m, respectively. After 300 min incubation of insulin at 65 °C and after 240 min incubation of lysozyme at 65 °C with a stirring rate of 150 rpm, about 20 μL of insulin and lysozyme from different vials was transferred to glass plate and air dried overnight at room temperature. AFM images were captured in the semi contact mode.

2.9. Circular dichroism (CD) measurements

All CD measurements were carried out on a JASCO CD spectropolarimeter (J-715), at 25 °C. A rectangular quartz cell with a path length of 0.1 cm was used. Owing to the high concentration of acetic acid (20% v/v), which precluded the CD measurements below 220 nm, the insulin solutions were diluted ten times from its working solution at the time of measurement. CD spectra were measured for insulin, prior, after 120 min, and after 300 min incubation, and for lysozyme, prior, after 60 min, and after 240 min incubation at fibril forming conditions. Each CD spectrum represents an average of three scans. The baseline of the sample CD spectrum was corrected by subtracting the CD spectrum of solvent. The spectra were recorded with a scan speed of 50 nm min^{-1}. The bandwidth and response time was 1.0 nm and 1 s, respectively.