Analytical Biochemistry 532 (2017) 83–86

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Thioflavin T fluorescence to analyse amyloid formation kinetics: Measurement frequency as a factor explaining irreproducibility

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ARTICLE INFO

Article history:
Received 11 April 2017
Received in revised form
9 June 2017
Accepted 12 June 2017
Available online 13 June 2017

Keywords:
Amyloid
Thioflavin T
Fluorescence
Islet amyloid polypeptide
Amyloid-beta peptide

ABSTRACT

The most frequent method to monitor amyloid formation relies on the fluorescence of thioflavin T (ThT). The present study reports a novel factor of irreproducibility in ThT kinetic assays performed in microplate. Discrepancies among kinetics of amyloid assembly, performed under quiescent conditions, were associated with the frequency of fluorescence measurement. Evaluating self-assembly of the islet amyloid polypeptide at short intervals hastened its fibrillization. This observation was confirmed by transmission electron microscopy, circular dichroism spectroscopy and 8-anilino-1-naphthalenesulfonic acid fluorescence. This effect, attributed to agitation during microplate displacements between fluorescence measurements, reinforces the importance of a better standardization in amyloid formation assays.

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Aggregation and tissue deposition of proteins into the form of amyloid fibrils are intrinsically associated with pathological states, including Alzheimer's disease, transthyretin amyloidosis and type II diabetes [1]. The causative association between amyloid formation and the pathogenesis has been demonstrated by compelling genetic and pharmacological evidence [1]. Nonetheless, therapeutic strategies to arrest protein aggregation and cell degeneration are still sparse. Thus, the identification of pharmaceutical inhibitors of amyloid formation is an active area of research. Moreover, understanding the molecular details of amyloid assembly is critical for the rational design of inhibitors. Therefore, evaluation of amyloid formation *in vitro* is important from both a mechanistic and a pharmaceutical perspective. The most commonly used approach to study kinetics of amyloid formation is the time-course measurement of thioflavin T (ThT) fluorescence [2]. ThT is a benzothiazole dye that exhibits a strong increase of its fluorescence quantum yield and a shift of its excitation spectrum upon binding to cross-β-sheet quaternary structure of amyloids [3–5]. Since the first study reporting over 50 years ago that ThT detects amyloids in *ex vivo* tissues [6], the ThT kinetic assay has become widespread for the mechanistic study of fibrillization [3,7]. Particularly, this assay can be performed in a microplate, allowing for high-throughput screening of amyloid modulators [8,9].

While fibrillization kinetic assays are important for the development of pharmaceuticals and mechanistic studies, polypeptide self-assembly is notoriously sensitive to numerous parameters, including concentration [10], temperature [10], buffer [11], surfaces [12], air-liquid interface [13], exogenous compounds [14–16] and agitation [17]. Accordingly, comparison of ThT kinetic assays between studies is particularly problematic owing to small differences in experimental conditions. We recently noticed that even when all these parameters are carefully maintained constant, discrepancies among kinetics of amyloid formation monitored by ThT fluorescence in microplates were still observed. These differences were associated with the frequency at which fluorescence is measured. Strikingly, intervals of ThT fluorescence measurement during amyloid assays performed under quiescent conditions vary significantly between studies [11,18–21] and the effect of this unstandardized, most often unreported, parameter has not been addressed so far. In this study, by using the islet amyloid polypeptide (IAPP), we depicted how amyloid formation is modulated by the measurement frequency in microplate.

IAPP is a 37-residue peptide hormone whose deposition into the pancreatic islets is associated with type-II diabetes [22]. Due to its high amyloidogenicity, amyloid assembly of IAPP under quiescent conditions occurs within a few hours, as observed by ThT

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http://dx.doi.org/10.1016/j.ab.2017.06.007
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fluorescence [23]. To specifically probe the effect of the frequency at which ThT fluorescence is measured on the fibrillization kinetic, the fluorescence of ThT was measured using intervals between 30 s and 1 h (for detailed materials and methods, please consult the supporting information). As shown in Fig. 1A, decreasing the interval between each measurement hastened amyloid assembly; *i.e.* a shorter lag-phase is observed at 0.5-min and 1-min intervals compared to 60 min. The times to reach half the maximum ThT fluorescence (t_{50}) were obtained by fitting the data to a Boltzmann sigmoidal and were used to calculate the lag-time (t_{lag}). Both t_{50} and t_{lag} increased proportionally with the measurement interval (Fig. 1B). In contrast, ThT fluorescence intensities at the plateau were not considerably different, except for the 60-min interval (Fig. 1B). We also confirmed this effect with a different microplate spectrophotometer (Fig. S1). Moreover, this effect persisted throughout a wide range of peptide concentrations (Fig. S2).

We next verified whether the differences in the fibrillization kinetics under quiescent conditions as a function of measurement interval correspond to actual IAPP conformational transitions. An amyloid assembly experiment in microplate and in presence of ThT was performed and at specific times, samples were analyzed by circular dichroism (CD) spectroscopy and transmission electron microscopy (TEM). When fluorescence was measured every 10 min, the random coil-to-β-sheet secondary conformational conversion associated with amyloid formation occurred after 5 h incubation (Fig. 1C). By TEM, no fibrils were observed after 3 h incubation whereas upon 5 h and 24 h incubation, fibrillar aggregates were detected (Fig. 1D). In sharp contrast, when fluorescence was measured every 1 min, the change in secondary structure was noticeable after 3 h (Fig. 1E) and the presence of fibrillar assemblies was confirmed by TEM (Fig. 1F). Interestingly, the morphology of the mature fibrils obtained after 24 h incubation was similar under both paradigms; *i.e.* 1-min and 10-min reading intervals.

According to studies showing that photoirradiation of ThT causes fragmentation of amyloid fibrils [24,25], we initially suspected that a similar mechanism involving ThT photodegradation could be implicated in these observations. To investigate whether or not ThT was directly involved in this effect, the concentration of ThT was initially varied by five orders of magnitude. By reducing the amount of ThT present in solution, any potential reactive intermediates generated by fluorescence measurement would have also been reduced. No differences were observed (Fig. 2A, Fig. 2B and Fig.S3). We then investigated if this effect could be related to an increase of local temperature around the ThT-fibril interface. Thus, assays were performed at 24 °C and 30 °C and variations between temperatures were negligible (Fig. 2C). To confirm that ThT excitation played no

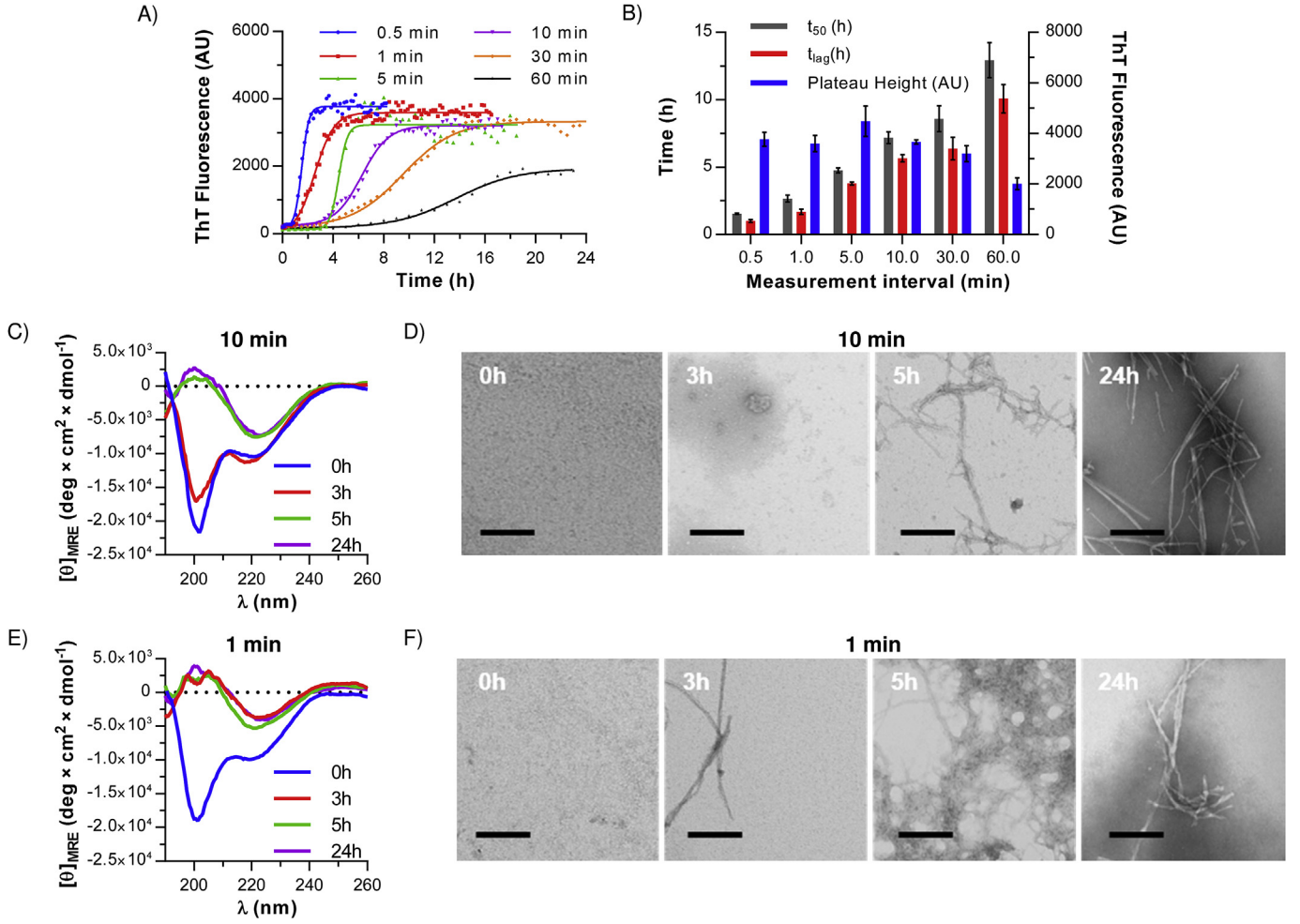

Fig. 1. Frequent ThT fluorescence measurements accelerate amyloid formation. (A) Kinetics of amyloid formation of IAPP (25 μM) measured by ThT fluorescence, with measurements taken every 0.5, 1, 5, 10, 30 and 60 min. (B) Kinetic parameters obtained from Boltzmann sigmoidal fit. (C,E) Circular dichroism spectra of samples taken from microplate assays showing changes in secondary structure for measurements taken at 10-min (C) and 1-min (E) intervals. (D,F) TEM images of samples taken from the microplate assays (in presence of 20 μM ThT) with 10-min (D) and 1-min (F) measurement intervals. Scale bar: 200 nm.

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