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Facile Methodology for Monitoring Amyloid-β Fibrillization

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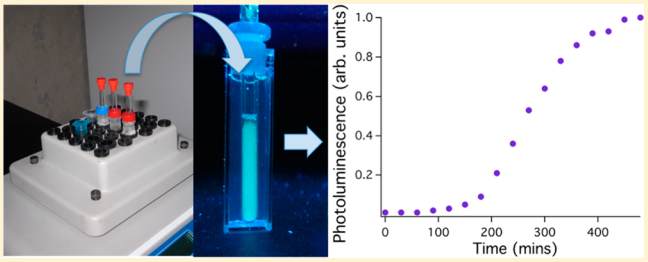
ABSTRACT: Amyloid-β (Aβ) is a peptide fragment that is prone to aggregate into large fibrils under physiological conditions. Many techniques have been developed to quickly monitor the transition from a primarily monomeric peptide into fibrils. Here we propose a novel method for both incubating and monitoring changes in Aβ aggregation by using modified NMR tubes, a microtube thermoshaker, and a fluorescence or UV−vis spectrometer. These NMR tubes are thin and cylindrical, which allows efficient heat transfer and orbital shaking. Our results demonstrate that our technique is both reliable and expedient when tracking Aβ fibrillization using fluorescence or turbidity assays, which presents an alternative for laboratories without specialized equipment for incubating peptide.



KEYWORDS: *Amyloid-β, fluoresence, fibrillization, turbidity, real-time assay*

Amyloid-β (Aβ) is a naturally produced peptide byproduct that can be found in the cerebrospinal fluid and brains of normal, healthy humans. The exact link between Aβ accumulation and Alzheimer's disease is unclear, but the amyloid cascade hypothesis suggests that the transition from monomeric forms of Aβ to oligomers and larger aggregates, such as fibrils, plays an important role in the progression of the disease.[1] The evolution of monomeric Aβ to aggregated forms is of great interest for potential treatments for Alzheimer's disease (AD), and numerous probes exist, such as Thioflavin T[2] (ThT), Congo Red,[3] and $[Ru(bpy)_2dppz]^{2+}$ (recently reported by our group)[4] that preferentially bind fibrillar forms of the Aβ peptide. ThT is a benzothiazole dye that displays minimal fluorescence in water, but greatly enhanced fluorescence when bound to amyloid aggregates.[5] The change in fluorescence of ThT is a property that is not unique to Aβ, but characteristic of many amyloid proteins such as insulin,[6] α-synuclein,[7] and hen egg white lysozyme,[8] among others.

Synthetic Aβ is commonly used in vitro to gain a greater understanding of how Aβ might function in vivo, having applications in the development of potential inhibitors or treatments,[9,10] novel detection methods,[4,11,12] or elucidation of aggregation pathways.[13−15] Detailed techniques for monitoring the aggregation of Aβ and other amyloid proteins in vitro have been reviewed by Nilsson.[16] Generally, an assay begins when lyophilized Aβ is dissolved in a pretreatment solvent such as aqueous NaOH[17] or hexafluoroisopropanol (HFIP)[18,19] to disrupt existing aggregates. The solvent is then removed or diluted into an appropriate buffer, then incubated for a set time, usually at 37 °C with orbital shaking. Real-time studies of aggregation are often adapted from the methods of Levine[2] and Naiki et al.[20] either using a microplate reader[21−24] or measuring in a cuvette with the probe directly added to the sample.[4,25] Alternatively, Aβ aggregation can be monitored using the aforementioned platforms, diluting an aliquot of Aβ in a solution with the probe.[26−29] Longer term experiments more interested in the presence of fibrils for further study generally seal the peptide solution in a microcentrifuge tube or glass vial and incubate for the desired time.[10,17,28,30]

We have found that although the techniques for monitoring Aβ aggregation have been well developed and described, other than using a microplate reader, most techniques require the transferring of the peptide solution from an incubator vessel (e.g., a microcentrifuge tube) to a cuvette. Over time this can result in significant loss of material due to evaporation or simply solvent adhering to the walls of a pipet tip or cuvette. If multiple samples are being tested the time required transferring the solutions and cleaning the cuvettes can be pressing, especially if the samples are being measured in real-time. On the other hand, monitoring Aβ fibrillization using microplate readers requires an instrument with fluorescence capabilities, orbital shaking, and bottom reading capabilities, since the microplate needs to be sealed on the top to prevent solvent evaporation. Interestingly, many microplate readers are equipped with linear, rather than orbital, shaking, which makes them unsuitable for reproducible Aβ incubation. Furthermore, obtaining a microplate reader with all the capabilities for monitoring Aβ aggregation can be expensive and they are never as sensitive as a standard spectrofluorometer.

In this paper, we report a novel methodology for growing and monitoring amyloid proteins reproducibly, and without the

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need of expensive or specialized instrumentation. A critical step in our techniques is the use of NMR tubes to incubate and monitor protein fibrillization. The advantage of this system is twofold: (1) it generates reliable and reproducible measurements within samples, and (2) it allows expedited measurements in a closed system that can be readily transferred between an incubator and a photoluminescence or UV−vis spectrometer. We suggest that this technique can allow any laboratory with spectrometry equipment to become active investigators in amyloid research with minimal additional investments.

DESCRIPTION OF THE TECHNIQUE

Materials.

1 NMR tubes (Wilmad-LabGlass, 528-PP-7)
2 Screw-cap microcentrifuge tube (Perfector scientific, 6550)
3 Standard spectrofluorometer cuvette, 1 cm walls with PTFE stopper in "ground type" joint (Starna cells, 23/Q/10)
4 Microtube thermoshaker (Boekel, 270500)

NMR tubes were cut to fit within the sample chamber of the fluorometer. The tube was cut such that it was as small as possible, but still able to be easily removed from the microcentrifuge tube or the spectrofluorometer cuvette (usually 5−6 cm long, Figure 1a). The PTFE cap of the cuvette and the plastic cap of the microcentrifuge tube were drilled through the middle, allowing a good fit of the NMR tube (Figure 1b, c). Care should be taken that the tube fits snugly in the hole. Tubes were incubated in microcentrifuge tubes that were wrapped with tape to sit firmly within the shaker chambers (Figure 1c, d). Microcentrifuge tubes were also filled with water to ensure even heating throughout the tube. For fluorescence or turbidity experiments, the samples were removed for a brief moment from the microcentrifuge tube and placed in the cuvette (Figure 1e, f). The cuvette was then transferred to a spectrofluorometer (for ThT assays) or to a UV−vis spectrometer (for turbidity assays) to obtain the spectra. The cuvettes were filled with water to avoid issues of water condensation forming on the inside walls of the cuvette. The tube caps were also marked with the orientation of the initial measurement, to ensure consistency throughout the experiment.

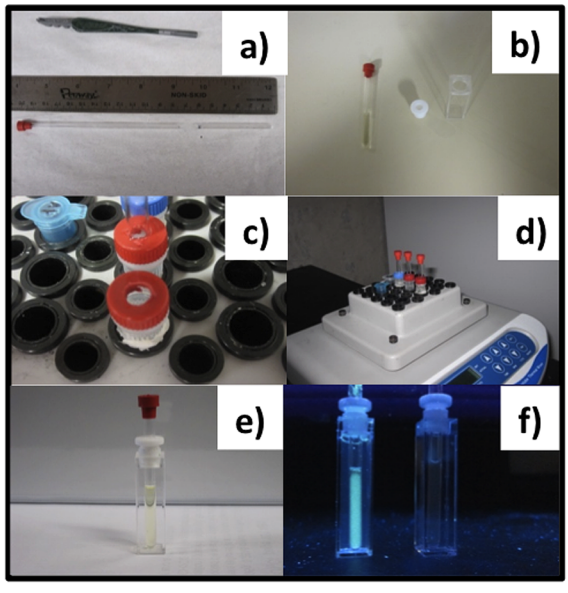

Figure 1. (a) NMR tube is cut to appropriate length; (b) tube along with cuvette and Teflon plug; (c) microcentrifuge tubes with a hole for holding the NMR tubes; (d) tubes are incubated in an orbital shaker with temperature control; (e) NMR tube within spectrometer cuvette; (f) NMR tube with aggregated protein and ThT under black light (left) and ThT only (right).

RESULTS AND DISCUSSION

The real-time Aβ fibrillization assay shows a typical sigmoidal curve as can be observed in Figure 2a. In addition to the increase in fluorescence of ThT, AFM was used to confirm the formation and uniformity of fibrils (Figure 2b). It was observed that the time to aggregation varied depending on the batch of amyloid prepared (data not shown). Nonetheless, minimal deviation was observed within samples of the same batch in terms of maximum fluorescence intensity and time to aggregation. The uniformity of our data testifies to the reproducibility of the fibrillization assays performed using this technique. This methodology could be a useful tool when evaluating the potential kinetic or thermodynamic inhibition of Aβ aggregation.[31] It is important to highlight that variations in the fibrilization rate from batch to batch are expected and have been studied by other groups.[17,27] These variations are due, among other things, to small changes in sample preparation and different initial peptide states. What we recommend to minimize this is to always compare the real-time fibrillization experiments with control samples from the same protein pool and incubated concurrently.

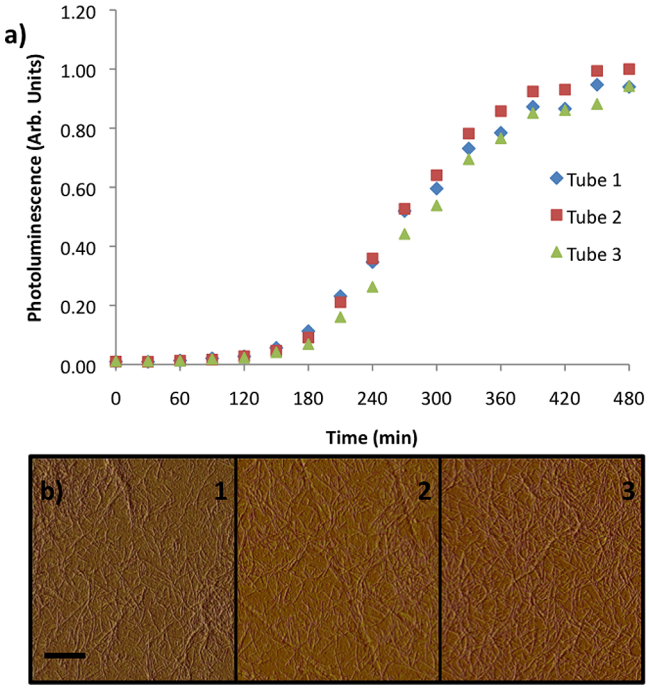

Figure 2. (a) Real time fluorescence assay with ThT (b) corresponding 10 × 10 μm AFM for each tube referenced in panel (a). Scale bar represents 2 μm.

We also sought to demonstrate other applications of this methodology by monitoring Aβ aggregation using UV−vis turbidity measurements. As Aβ aggregates into insoluble fibrils,

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the scattering of transmitted light increases significantly.[32,33] Figure 3 shows a typical sigmoidal curve representative of Aβ

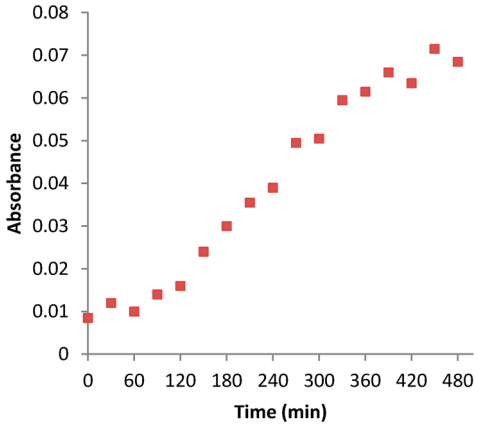

Figure 3. Real-time turbidity assay of Aβ measured at 400 nm.

aggregation. Alternative and rapid methods of monitoring Aβ aggregation would be desirable when working with inhibitors that are intrinsically fluorescent such as curcumin or other polyphenols that may potentially bias an assay. Furthermore, this methodology can be applied to monitor fibrillization in the presence of molecules that inhibit ThT binding to Aβ fibrils.[31,34]

CONCLUSION

Great progress has been made in the study of Aβ since it was first characterized by Glenner and Wong.[35] A diversity of techniques have been utilized to improve our understanding of this important peptide and its aggregation. In this paper, we have presented a convenient methodology to perform real-time fibrillization assays. Our experiments indicate this is a reliable and rapid technique for monitoring in vitro fibril formation. Furthermore, the instrumentation necessary is common in many laboratories, making this a low cost alternative for studying Aβ aggregation.

METHODS

Preparation of Aβ. Bulk unpurified amyloid-β (Aβ) 1−40 (lot #20529) was purchased from 21st Century Biochemicals. The crude peptide was desalted and concentrated using Sartorius Stedim Vivaspin 15r centrifugation filters. The desalted solution was lyophilized using a Labconco Centrivap Concentrator and either immediately purified by reverse phase HPLC on a Waters Xbridge Prep C18 column or stored at −20 °C for later use. Reconstituted peptide solution (200 μL) was then run on a gradient of A (H_2O, 0.1% TFA) and B (acetonitrile, 0.1% TFA) at 40 °C (80% A to 40% A over 40 min at 3 mL/min). The retention time of Aβ 1−40 is ∼22 min. The purified peptide is then verified with ESI-mass spectrometry, lyophilized, and stored at −20 °C.

Preparation of Fibrillar Aggregates and Real-Time Assay. Fibrils from Aβ were prepared from a purified lyophilized powder by reconstituting in a minimal amount of aqueous NaOH solution.[17] Freshly dissolved peptide was then sonicated for 2 min in a bath sonicator and subsequently filtered through 0.2 μm centrifuge filters (VWR) for 10 min at 5000*g*. The filtered solution was then diluted to the desired concentration in PBS (100 mM sodium phosphate, 300 mM NaCl, pH 7.4). The concentration was verified using an extinction coefficient of 1280 M^{-1} cm^{-1} at 280 nm using a Shimadzu 2450 UV−vis spectrophotometer. Typical Aβ concentrations for these experiments were 60 μM. A concentrated solution of ThT was then added to the Aβ solution to obtain a 10 μM concentration of the dye. 400 μL of the peptide solution was then aliquoted into NMR tubes. Measurements were taken every 30 min as described below. Prior to every measurement each NMR tube was briefly vortexed (∼1−2 s). The tubes were incubated as shown in Figure 1, at 37 °C and 700 rpm.

Fluorescence Measurements. All steady-state fluorescence measurements were taken on a Horiba-Jobin Yvon Fluorolog 3 instrument. ThT was excited at 440 nm, and right angle emission was measured from 460 to 520 nm with 1 nm slit widths. Both emission and excitation were corrected for instrument dependent effects. The fluorescence intensity of ThT at 484 nm was used to monitor the transition of Aβ monomers to fibrils.

Turbidity Assay. An NMR tube filled with PBS solution as referenced above was baselined and samples were measured every 30 min at 400 nm on a Shimadzu 2450 UV−vis spectrophotometer.

Atomic Force Microscopy. Twenty microliters of peptide solution was dropped onto a freshly cleaved mica surface and allowed to adhere for 5 min. After 5 min the mica discs were dried on a spin coater at 3000 rpm and washed 3 times with 20 μL of H_2O. Images were acquired at 512 lines of resolution at 1 Hz on a Veeco Multimode atomic force microscope.

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Author Contributions

N.P.C. and A.A.M. designed the research. N.P.C. performed the research. N.P.C. and A.A.M. analyzed the data. N.P.C. and A.A.M. wrote the paper.

Notes

The authors declare no competing financial interest.

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