SHIMADZU
Excellence in Science

Application News

No. A620

Spectrophotometric Analysis

# Evaluation of Amyloid-β Aggregation by RF-6000 Spectrofluorophotometer

Amyloid-β is a peptide that consists of approximately 40 amino acid residues. Amyloid fibrils (fibrous aggregates), which occur as a result of the formation of parallel β sheets by intermolecular association of amyloid-β, are the main component of the senile plaques (amyloid plaques) seen in the brains of Alzheimer's disease patients. Moreover, the formation mechanism and structure of amyloid fibrils have attracted great interest, as amyloid fibrils are also implicated in other neurodegenerative diseases, including Parkinson's disease [1].

Fluorometric analysis using Thioflavin T (ThT), a fluorescent dye used as a marker for amyloid aggregation, is a suitable technique for evaluating amyloid-β aggregation. In this experiment, the formation process of amyloid fibrils was monitored by measuring the fluorescence intensity of ThT and the Rayleigh scattering intensity of the sample solutions, which is proportional to turbidity, at regular time intervals.

Although ThT emits almost no fluorescence when it exists in a free state in a solution, it exhibits extremely strong fluorescence (excitation wavelength: 440 nm, fluorescence wavelength: 482 nm) upon binding with amyloid fibrils [2]. Fig. 1 shows the structural formula of ThT.

R. Fuji, K. Maruyama, S. Iwasaki

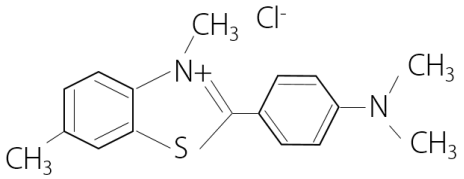



**Fig. 1 Structural Formula of ThT**

The structure of ThT consists of a hydrophobic terminal, in which a dimethylamino group is bonded to a phenyl group, and a high polarity benzothiazole group that includes polar N and S. As a result of this combination of a polar region and hydrophobic region, ThT molecules readily form micelles in aqueous solutions, and the polar region containing positively charged N is exposed to the solvent. Based on this, it has been suggested that the N in the ThT and hydroxyl groups in the tissue structure form hydrogen bonds, and these ThT dye molecules bind specifically to amyloid and other tissue structures [3].

In this experiment, phenol red was used as an inhibitor. Phenol red inhibits the progress of amyloid-β aggregation by improving the solubility of amyloid-β (protofibrils) in the initial stage of aggregation [4]. Fig. 2 shows the structural formula of phenol red.

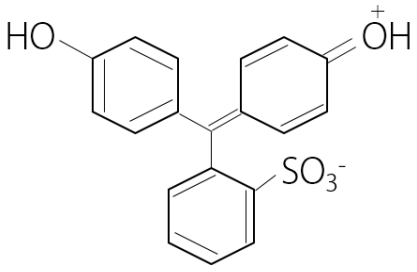



**Fig. 2 Structural Formula of Phenol Red**

## Experimental Procedure

Here, amyloid-β (human, 1-40) was used. The amyloid-β pretreatment procedure, preparation of the various reagents, and measurement method are presented below.

[Pretreatment of amyloid-β [5]]

1. Freeze-dried amyloid-β was dissolved in a 2 mM NaOH aqueous solution at a ratio of 1 mg of amyloid-β to 1 ml of the NaOH solution, and the pH was adjusted up to 10.5 using a 0.1 M NaOH aqueous solution.
2. After sonication for 1 min at room temperature, the solution was freeze-dried, and the dried sample material was stored at -80 °C.

[Preparation of reagents]

Solution A: 50 mM Tris / 150 mM NaCl buffer
1 M Tris-HCl and 3 M NaCl solutions were mixed, diluted, and adjusted to pH 7.2. The buffer was then stored at 4 °C.

Solution B: 200 µM ThT solution [dye marker for amyloid aggregation]
A 200 µM ThT solution was prepared by mixing ThT and solution A, followed by filtration with a 0.2 µm syringe filter.

Solution C: Amyloid-β solution
Solution A at 4 °C was added to the pretreated amyloid-β, which was adjusted to approximately 12 µM and completely dissolved by sonication for 1 min. Following centrifugal separation (10,000 rpm, 5 min) at 4 °C, the supernatant was sampled and stirred.

Solution D: 200 µM phenol red solution [inhibitor]
The phenol red solution was prepared by mixing phenol red and solution A.

[Measurement method]

1. Two types of amyloid-β solutions (final concentration: approx. 10 µM) with and without an inhibitor were prepared by mixing the various reagents, and were set in a thermostatic chamber at 37 °C.
   - With inhibitor: Prepared by mixing solutions B : C : D at a ratio of 2 : 17 : 1.
   - Without inhibitor: Prepared by mixing solutions B : C : A at a ratio of 2 : 17 : 1.
2. 100 µL of the sample was introduced in a microcell, and the sample was stirred by shaking the cell immediately before the measurement. Fluorescence measurements were carried out at 20 min intervals over a total period of 5 h.

## Instrument and Measurement Conditions

Fig. 3 shows the appearance of the RF-6000 spectrofluorophotometer, and Table 1 shows the measurement conditions. Highly sensitive measurement is possible with the RF-6000 due to its excellent S/N ratio of 1,000 or more (RMS), which is the highest in its class.

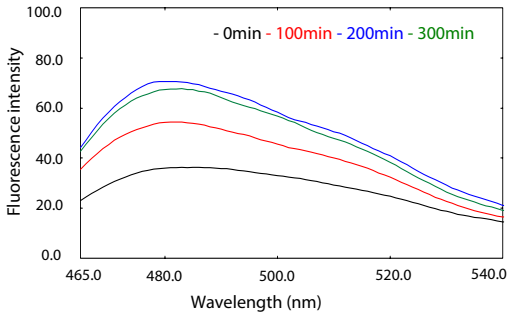
**Fig. 3 RF-6000 Spectrofluorophotometer**

**Table 1 Measurement Conditions**

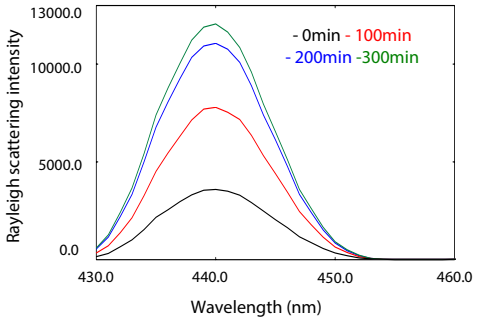
| | |
|---|---|
| Instrument | : RF-6000 spectrofluorophotometer |
| Excitation wavelength | : 440 nm |
| Fluorescence wavelength range | : 430 - 550 nm |
| Scan speed | : 600 nm/min |
| Sampling pitch | : 1.0 nm |
| Bandwidth | : Ex 5 nm, Em 10 nm |
| Sensitivity | : Low |

## Measurement Results

Fig. 4 (a) and (b) show the fluorescence spectra of the amyloid-β solution (without inhibitor) after 0, 100, 200, and 300 min. The ordinates in (a) and (b) show the fluorescence intensity and Rayleigh scattering intensity, respectively. In both cases, the intensity increases with time.




(a) Fluorescence intensity




(b) Rayleigh scattering intensity

**Fig. 4 Fluorescence Spectra of Amyloid-β Solution (Without Inhibitor)**

Fig. 5 shows the fluorescence intensity of 482 nm at each measurement time, and Fig. 6 shows the Rayleigh scattering intensity of 440 nm at each measurement time. From Fig. 5, the fluorescence intensity of the sample without the inhibitor increased with the passage of time, but a similar increase in fluorescence intensity was not observed in the sample with the inhibitor. In Fig. 6, the Rayleigh scattering intensity of the sample without the inhibitor increased remarkably with time, suggesting that turbidity increased as the amyloid-β aggregated and formed amyloid fibrils. In contrast, no increase in Rayleigh scattering intensity was observed in the sample with the inhibitor.

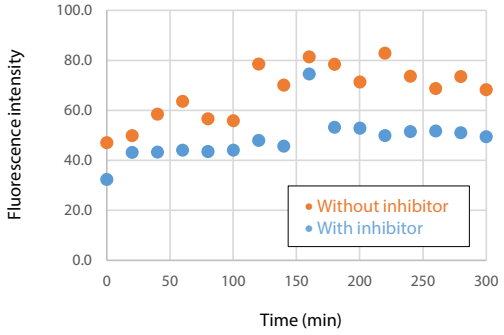



**Fig. 5 Fluorescence Intensity of 482 nm at Each Measurement Time**

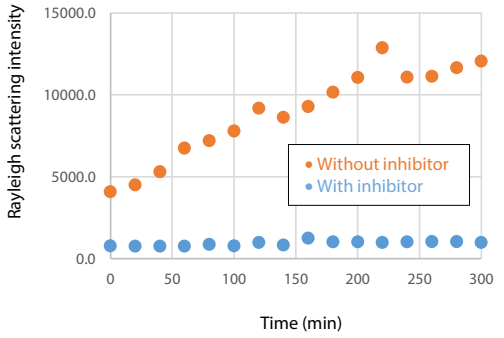



**Fig. 6 Rayleigh Scattering Intensity of 440 nm at Each Measurement Time**

## Conclusion

The changes in fluorescence intensity and Rayleigh scattering intensity accompanying aggregation of amyloid-β were measured with a Shimadzu RF-6000 spectrofluorophotometer. The intensity behavior differed depending on whether an inhibitor was used or not. When the inhibitor was not used, the results revealed that both fluorescence intensity and Rayleigh scattering intensity increased due to aggregation of amyloid-β with elapsed time.

<References>
(1) Sean D. Moran and Martin T. Zanni, J. Phys. Chem. Lett. 2014, 5, 1984-1993
(2) Shinya Sugimoto, Kagaku to Seibutsu. 2017, 55(8), 573-579
(3) Ritu Khurana et al., Journal of Structural Biology. 2005, 3, 151, 229-238
(4) Chun Wu et al., Biophys J. 2006, 91(10), 3664-3672
(5) Youcef Fezoui, Amyloid: Int. J. Chin. Invest. 2000, 7, 166-178

First Edition: Jun. 2020

SHIMADZU

Shimadzu Corporation

www.shimadzu.com/an/