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Nile red compensates for thioflavin T assay biased in the presence of curcumin

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ABSTRACT

Amyloid fibrillary aggregates, which are gradually formed under predisposing conditions in the human body, are the main sign of conformational diseases such as type 2 diabetes, Alzheimer's disease and Parkinson's disease. How the aggregates are formed, what characteristics they have, and how their formation can be inhibited, have been targeted in many studies thus far. In all of such studies, thioflavin T (ThT) fluorescence assay is used to qualify the amyloid aggregates and the inhibitory effect of polyphenolic small molecules. Among the proteins with the potential of the aggregation in vitro, hen egg white lysozyme (HEWL) is much used, and on the other hand, curcumin, a known polyphenol, has a potential to inhibit the aggregation process. However, whether curcumin affects the aggregation process or not, ThT fluorescence is biased in the presence of curcumin because it displays an enhanced absorption at the excitation wavelength of ThT. To address this problem and to qualify the aggregation more accurate, it is recommended to use Nile red (NR) anisotropy as an alternative fluorescence-based method which considers molecular sizes, not emissions. Since NR, as bound to amyloid fibrils, fluoresces significantly beyond the range of curcumin, NR anisotropy can further minimize the interference effect of curcumin.

1. Introduction

Amyloid fibrillary aggregates as the main cause of conformational diseases, including Alzheimer's disease, Parkinson's disease and type 2 diabetes, are of quaternary protein structures, which gradually formed and accumulated in different areas of the human body due to misfolding of some special polypeptides under predisposing conditions [1–3]. To study the aggregates, amyloidogenic proteins (either of natural or model type) must already have been exposed to fibrillation conditions in vitro [4,5]. Among the model proteins, hen egg white lysozyme (HEWL) is commonly used because it is a readily available protein, its fibrillation process can easily be under controlled, and properties of its fibrillar form resemble that of native amyloidogenic protein such as amyloid-β, α-synuclein, and tau. HEWL fibrillation requires the incubation at high temperature (54–70 °C) at acidic pH (2 − 3) so that under such conditions, the transformation of HEWL monomeric solution to the amyloid one occurs in several days [6–9].

Several methods have been developed to identify amyloid fibrils, including fluorescence assays, circular dichroism (CD) spectroscopy, and imaging by scanning or transmission electron microscopy. Due to their simplicity and availability, the fluorescence-based methods (e.g. thioflavin T (ThT) fluorescence assay) are usually the first choice. Upon binding with amyloid fibrils, ThT undergoes a conformational change and fluoresces significantly and thus it can be used to monitor amyloid-related structural changes in real time [10–13].

Since amyloid species were introduced as entities with cytotoxic properties, various strategies have developed to inhibit the formation of them [14]. Among the strategies, the use of various polyphenolic small molecules has been received the attention of many researchers all around the world. It has been shown that the size and population of mature amyloid fibrils formed in the presence of the polyphenols are much less than those in the solution lacking such small molecules [15–19].

Of the polyphenols, curcumin (Cur) is a natural polyphenol from Curcuma longa that its inhibitory potential on the fibril formation has been reported in several papers [20–26]. The most common protocol is that two samples, a control sample containing only the protein monomeric solution and another containing the same protein mixed with Cur, are incubated simultaneously under the same fibrillation condition. Once the fibrillation time is over, a given volume of each sample is mixed with the ThT solution and the fluorescence emission intensity is subsequently measured. The results have always shown that the fluorescence intensity of ThT is lower in samples containing Cur.

Although Cur inhibitory strength has been verified by some non-

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Table 1
Solutions of ThT and Cur for absorption and emission measurements.

Name	Description
ThT /DMSO	ThT solution (25 μM) prepared in DMSO
ThT /PhB	ThT solution (25 μM) prepared in PhB with 0.1% DMSO
L_a + ThT	ThT solution (25 μM) containing 20 μM of L_a
Cur /DMSO	Cur solution (25 μM) prepared in DMSO
Cur /PhB	Cur solution (25 μM) prepared in PhB with 0.1% DMSO
L_a + Cur	Cur solution (25 μM) containing 20 μM of L_a
L_a + (Cur + ThT)	A mixture of ThT and Cur (each 25 μM) containing 20 μM of L_a
NR /DMSO	NR solution (25 μM) prepared in DMSO
NR /Gly	NR solution (25 μM) prepared in the glycine buffer with 1% DMSO
L_a + NR	NR solution (25 μM) containing 20 μM of L_a
L_a + (Cur + NR)	A mixture of NR and Cur (each 25 μM) containing 20 μM of L_a

fluorescence methods such as circular dichroism (CD) spectroscopy and transmission electron microscopy (TEM), it was shown in 2009 that ThT fluorescence assay is biased in the presence of Cur. As reported, increasing the concentration of Cur (from 0 to 100 μM) caused ThT fluorescence to be dropped off due to a phenomenon is known as inner filter effect [29]. Inner filter effect is one of the main sources of errors in fluorescence experiments such as quenching, so that in an emission measurement experiment, the apparent decrease in emission intensity may be the result of the absorption of incident light by a species other than the intended primary absorber (primary inner filter effect) and/or the absorption of emitted light of the primary absorber (secondary inner filter effect) [27,28]. As reported in the study [29], both kinds of inner filter effect occur in the presence of Cur so that it absorbs the incident light and thus blocks the fluorescence of ThT and also prevents the emission of excited ThT. Our study, however, shows that only one of them, the secondary inner filter effect, occurs.

The study in 2009 has only performed on proteins "reduced and carboxymethylated κ-casein (RCMκ-CN) and amyloid-β (Aβ)" [29]. Can these results be applied to other amyloidogenic proteins such as HEWL?

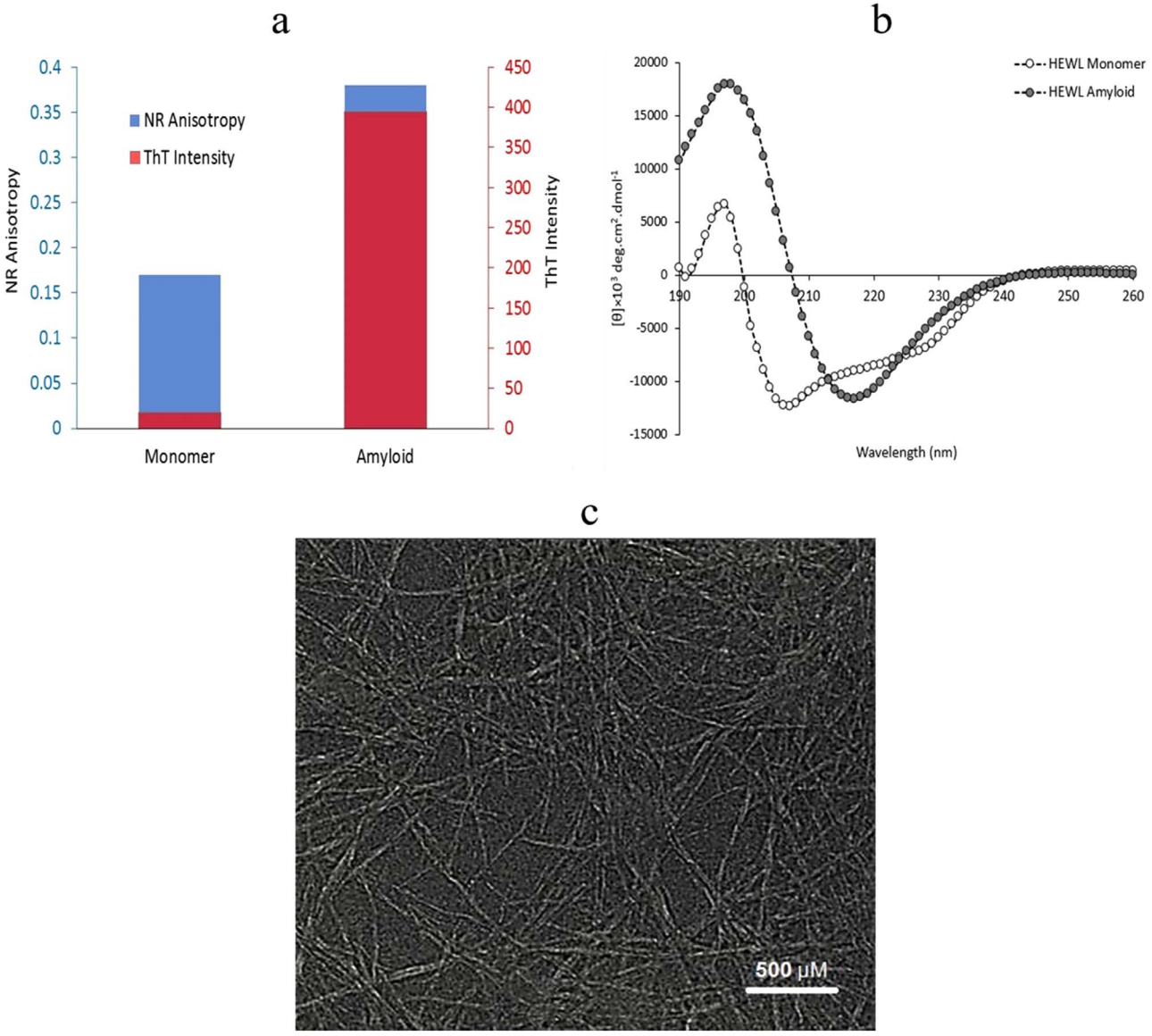

Fig. 1. Characteristics of HEWL amyloid fibrils. (a) The simultaneous comparison of ThT fluorescence and NR anisotropy of HEWL amyloid fibrils with those of HEWL monomers. (b) CD spectrum of HEWL amyloid fibrils with a characteristic peak at 218 nm which confirms the presence of the fibrils in the solution. (c) FE-SEM image of such fibrils.