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Specific Binding of β_2-Microglobulin with Trypan Blue

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Several staining methods have been developed to monitor protein fibril formation. Two widely used dyes that are now utilized in standard staining assays are Congo red and Thioflavin T (ThT). However, non-specificity, false negative results and a requirement for expensive instrumentation have precluded the use of these dyes in the characterization of amyloidogenic proteins. In this study, we developed a simple method to follow specific binding of β_2-microglobulin (β_2m) fibrils using UV-visible (Vis) spectroscopy with the Trypan blue (TB) dye. The use of UV-Vis spectroscopy as a technique for amyloid fibril demonstration serves as an advantage due to the availability of the instrument in most laboratories. Binding of β_2m fibrils was achieved by combining a solution of TB with a concentrated fibril solution followed by UV-Vis spectroscopy. Here we observed a significant shift of the λ_{max} towards a longer wavelength when TB specifically binds with the fibrils. Also, the observed increase in absorbance upon binding of TB was dependent on the amount of fibrils. This new and simple assay adds to the variety of staining methods which may potentially be used to analyze other protein fibrils like the Aβ in Alzheimer's disease and the prion protein in transmissible spongiform encephalopathy.

Key words —— β_2-microglobulin, Trypan blue, dialysis-related amyloidosis, amyloidosis

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INTRODUCTION

Specific aggregation of several proteins into long, non-covalent, and extra-cellularly deposited amyloid fibrils leads to the pathological disorder known as amyloidosis. This disease manifests through the deposition of soluble proteins into insoluble fibrils having β-pleated sheet structures.[1)] One such protein that has the susceptibility to form fibrils is β_2-microglobulin (β_2m), the light chain of the type I major histocompatibility complex. β_2m is a 12 kDa protein arranged in a beta sandwich motif (Fig. 1a). Its structure is stabilized by hydrophobic interactions and a single disulfide bond.[2–4)] In patients undergoing long-term hemodialysis, β_2m tends to aggregate into fibrils and form amyloid plaques. These fibrils are characterized by a cross-β structure where the β-strands are perpendicularly oriented to the axis of polymeric fibril. The cross-β structure of fibrils is a common characteristic of amyloidogenic proteins such as the Aβ protein of Alzheimer's disease and the prion protein associated with transmissible spongiform encephalopathies and Creutzfeld-Jacob disease.[5–7)]

Studies have been made on the various conditions that affect the propensity of β_2m to aggregate. β_2m lacking six residues in the N terminus has been shown to increase amyloidogenicity, and has been found *ex vivo* together with other truncated species.[6)] The reduction of the lone disulfide bond of β_2m, under acidic and high ionic strength conditions, has been shown to lead to the formation of short curvilinear immature fibrils.[7)] However, the complete mechanism of β_2m fibril formation is still unknown.

Staining methods are the primary tool for following β_2m fibrillogenesis. Congo red and Thioflavin T (ThT) are the most universally used dyes for this purpose. Congo red is an azo dye, which specifically binds to the β-pleated sheet fibril and induces a green birefringence under a polarization microscope. ThT, on the other hand, is believed to bind with the quaternary structure of fibrils rich in β-pleated sheets.[8–10)] In this study, we propose a simple UV-visible (Vis)-based assay that uses Trypan blue (TB) (Fig. 1b), another diazo dye, as a specific indicator of β_2m fibril formation. UV-Vis spectroscopy, a widely available detection tool was used to monitor the binding of TB to β_2m fibrils.

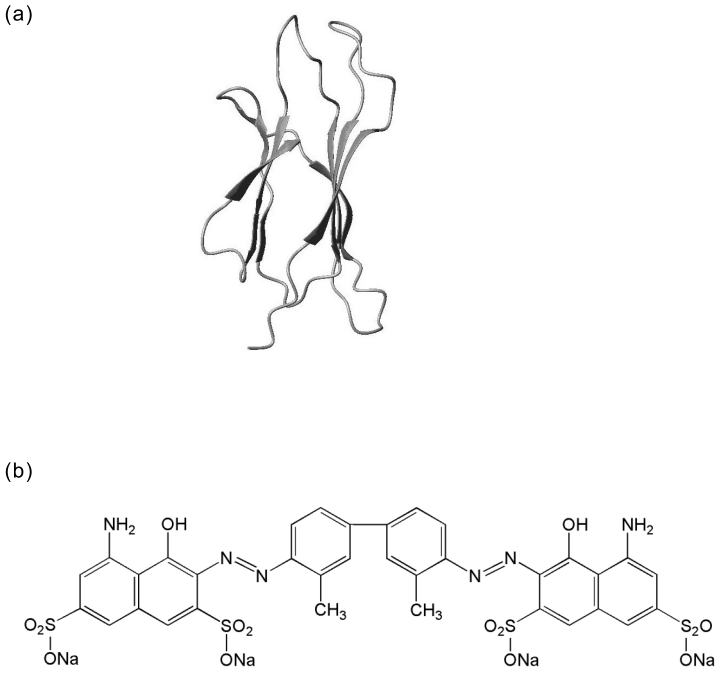


Fig. 1. Structure of β_2m and TB

(a) β_2m is the major constituent protein of Dialysis-Related Amyloidosis (DRA). β_2m consists of 99 amino acid residues, which forms 7 β-pleated sheets. (b) TB is a symmetrical diazo dye similar in structure to the widely used amyloid dye, Congo red.

MATERIALS AND METHODS

Purification of Recombinant β_2m —— Recombinant β_2m was obtained from a yeast (*Pichia pastoris, P. pastoris*) expression system that over expresses β_2m with an additional four residues (Glu-Ala-Tyr-Val) at the N-terminus.[11] Induction of the protein was made using methanol after 3 days of culture. The supernatant which contains the protein was desalted using Sephadex G-50. The sample was then added to a Sephacryl 200 column and protein was eluted using 10 mM phosphate buffer, pH 7.5. Active fractions were then pooled and applied to a DEAE-Sepharose equilibrated with a 20 mM Tris–HCl, pH 8.5 and elution of β_2m followed by a NaCl gradient. Purity of the protein was checked with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Sample Solution Preparation for Dye-Binding Experiments —— All chemicals used were of analytical grade. As stock, a solution of 200 μM β_2m in 50 mM citrate buffer, pH 2.5, and 100 mM KCl at 4°C was prepared. To prepare the seeded solution of β_2m, 10 μl of prepared fibrils were added to 1 ml of 25 μM monomeric β_2m. Hereafter, these two solutions will be referred to as the β_2m monomer and seeded/fibril solutions. Resulting solutions were then used for the TB binding studies.

Preparation of Fibril Solution —— β_2m amyloid fibrils were generated by the fibril extension method established by Naiki *et al.*,[12–15] wherein extension of seed fibrils was made by addition of the monomeric protein at pH 2.5 and 37°C, followed by fluorometric analysis by ThT. Fibrils were prepared by addition of sonicated 50 μl concentrated β_2m seeds to 1 ml of 100 μM monomeric β_2m. Ten microliters of 0.1%(w/v) NaN_3 may be added to the solution as preservative. The resulting solution was incubated at pH 2.5 and 37°C for up to 3 hr. Fibril formation was followed and observed by using the ThT Assay.

ThT Assay —— Thioflavin staining is widely used in the study of *in vitro* fibril formation. ThT is easily quenched by light thus, appropriate measures should be taken. β_2m fibril solution incubated at pH 2.5 was kept at 20°C throughout the experiment. An aliquot of 15 μl of the β_2m fibril solution was thoroughly mixed with a 3.0 ml solution of 5 μM ThT in 50 mM glycine-NaOH buffer (pH 8.5). Fluorescence was measured using a Shimadzu RF-5301 spectrofluorometer. Excitation and emission wavelengths were set at 445 and 485 nm, respectively. Fluorescence was measured at different time points upon incubation of the β_2m solution at 37°C.

TB Binding with β_2m —— A solution of 0.2%

TB (Invitrogen) in 90% phosphate-buffered saline (0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl) at pH 7.4 and 10%(v/v) ethanol was prepared. The stock solution was filtered using Whatman 0.2 μm nylon membrane filters to remove micelles of TB.

Test solutions of TB + β_2m monomer were prepared by keeping a 1 TB: 2 β_2m concentration ratio. Varying amounts of TB were added in the cuvette to give 20-, 40-, 60-, 80-, and 100-μM of TB. Corresponding amounts of β_2m were added to the cuvette to follow the aforementioned concentration ratio. The test solutions were scanned for its maximum absorption wavelengths. Solutions of the same TB concentration containing seeded β_2m fibrils instead of monomeric β_2m, and control samples (TB alone) were also subjected to the assay.

In addition, fibril extension studies using a reaction mixture of TB and β_2m fibrils were performed to observe changes in absorbance with time of the incubated reaction mixture. A solution of 60 μM TB was mixed thoroughly with 50 μM of the β_2m fibril solution in a cuvette. Absorbance readings were taken of the fibril solution at different time points upon incubation at 37°C.

Various amounts of both fibril and monomer were also monitored for differences in absorbance against a fixed concentration of the dye. A solution of 60 μM TB was mixed with solutions of β_2m monomer ranging from a concentration of 10 to 50 μM. Likewise, different amounts of β_2m fibrils (60 to 140 μl) were mixed with the same concentration of TB, and subjected to the same assay.

RESULTS

ThT Assay

A standard method used to monitor fibril formation is the ThT assay. Shown in Fig. 2 is the fluorescence intensity of β_2m at increasing incubation periods (from 0, 30, 60, 90, 120, 150, to 180 min) at 37°C. The obtained readings clearly show that fibril formation increases with longer incubation of the protein.

TB Assay

The TB assay developed monitors the absorbance at different concentrations of the dye. Figure 3 illustrates the absorbance spectra of TB alone, TB and β_2m monomer, and TB and β_2m fibrils. At various concentrations of a control reaction carried out in the absence of β_2m monomer or fibril, no significant shift in the absorption maxima was observed (Fig. 3a). Applying the 1 TB: 2 β_2m concentration ratio to the TB + β_2m monomer samples, negligible changes in the λ_{max} were likewise achieved (Fig. 3b). However, with the TB and β_2m fibril samples, a significant shift to a higher wavelength was detected (Fig. 3c). Table 1 summarizes the maximum absorption wavelength readings from the proposed TB assay obtained at different concentrations of TB.

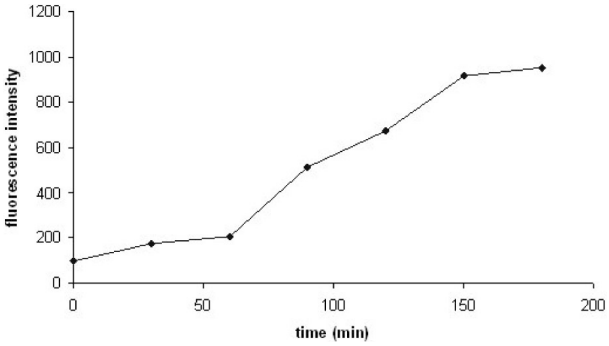


Fig. 2. Formation of β_2m Fibrils as Detected by the ThT Assay
Amyloid fibril formation was formed by the fibril extension method with monomeric β_2m (100 μM) and 50 μl concentrated seed fibrils at pH 2.5 and 37°C. The reaction was monitored by fluorometric analysis with 5 μM ThT in 50 μM glycine-NaOH, pH 8.5. Fibril formation is indicated by the continuous increase in fluorescence in less than 200 min.

The effect of varying incubation time (from 0, 180, to 270 min) on the assay performance is shown in Fig. 4. A solution containing 50 μM of incubated β_2m fibrils was examined for absorbance changes with 60 μM TB at different time points. It was observed that as the solution of the β_2m fibril was incubated through prolonged time periods, the absorbance at the maximum absorption wavelength increases.

TB assay results by adding varying concentrations of β_2m monomer and β_2m fibrils to a fixed concentration of TB are shown in Fig. 5a and 5b, respectively. No significant increase in the absorbance measurements or shift in the maximum absorption wavelength was observed for the β_2m monomer. On the other hand, a slight increase in the absorbance measurements and a spectral shift was observed when different amounts of β_2m fibrils (60–140 μl) were added to a fixed concentration (60 μM) of TB in solution. It should be noted though, that the amount of β_2m fibrils in solution should be greater than the amount of the TB.

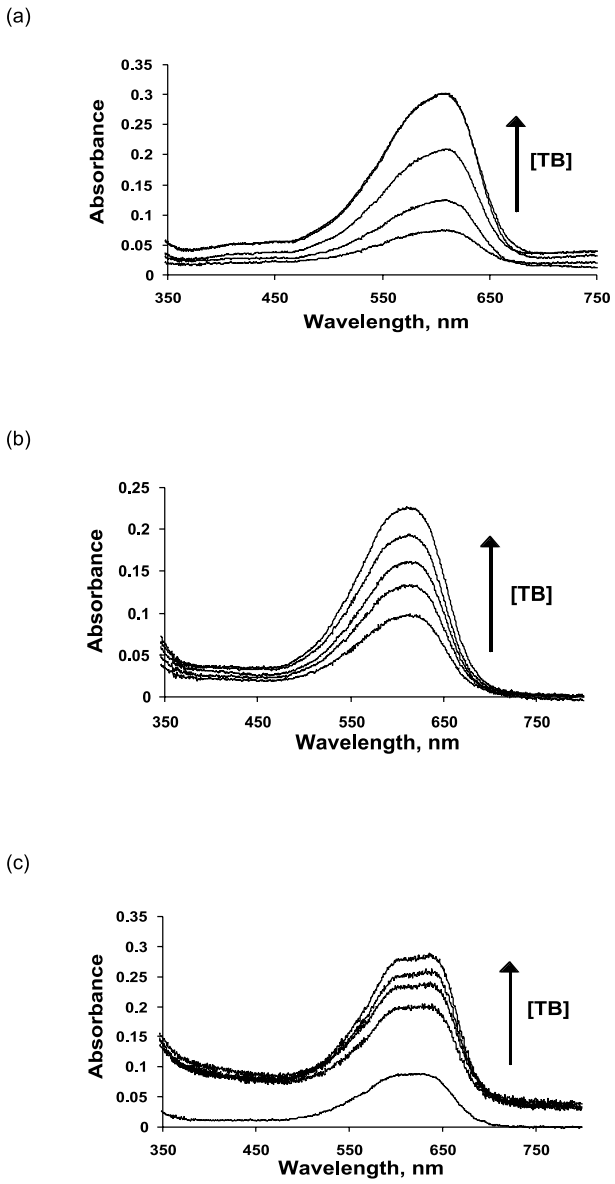


Fig. 3. Absorption Spectra of (a) TB Alone, (b) TB + β_2m Monomer, and (c) TB + β_2m Fibril

Binding of the β_2m fibril with TB exhibited a red shift compared to the non-fibrillar monomeric β_2m and TB. Negligible shifts in absorption maxima for the control samples (TB alone) and monomeric solution indicated that the spectral shift in the β_2m fibril and TB solutions were due to formation of the fibril. Increase in the absorbance measurements were observed as one increases the concentration of TB (20, 40, 60, 80, and 100 μM).

DISCUSSION

β_2m Fibril Formation

Native β_2m is stable at neutral pH, but is not prone to fibril formation even after long periods of incubation. Thus, an acidic pH environment is necessary for *in vitro* β_2m fibril formation. β_2m fibrils may be formed *in vitro* by incubation of recombinant wild-type β_2m at low pH and high ionic strength. Acidification below pH 5 ensures formation of fibrils. At pH 2.5 and at low ionic strength (50 mM), the protein adapts a polymeric state of long and straight fibrils. At pH 1.5–5.0 and high ionic strength (400 mM), fibrils formed are of curved morphology. In addition to these, protofibrillar structures are said to be formed initially before the assembly into β_2m fibrils. These structures are formed at pH 3.6.[1,16]

The conditions applied in the experiments follow the established set of conditions for β_2m fibril formation.[15] β_2m was dissolved in citrate buffer with 100 mM KCl at pH 2.5. This monomeric solution was seeded with sonicated β_2m fibrils. To accelerate fibril formation, the concentration of the β_2m solution was increased from 25 to 100 μM. The monomeric solution of β_2m would form fibrils upon incubation at 37°C, and may be detected using ThT.

ThT Assay

ThT may exhibit specific binding to amyloid fibrils hence it is extensively used for binding experiments. Its versatility is due to its rapid adherence to the fibrils and accompanied increase in fluorescence at 485 nm when excited at 455 nm.[16] Studies show that ThT interacts with the quaternary structure of the β-pleated sheet fibril, and the binding is not dependent on any peptide sequence.[9,17] Binding of ThT to β_2m fibrils induces a spectral shift (both emission and excitation) which may be used as an indicator for fibril formation.[18]

The fluorescence intensity was observed to increase with longer incubation of β_2m seeded solu-

Table 1. Maximum Absorption Wavelengths of TB and β_2m Solutions

[TB], μM	λ_{max}, nm TB Alone	λ_{max}, nm TB + β_2M Monomer	λ_{max}, nm TB + β_2M (with Fibril)
20	610.50	613.50	630.00
40	608.50	613.50	639.50
60	611.00	614.50	637.50
80	606.00	613.00	639.50
100	610.00	609.50	636.50

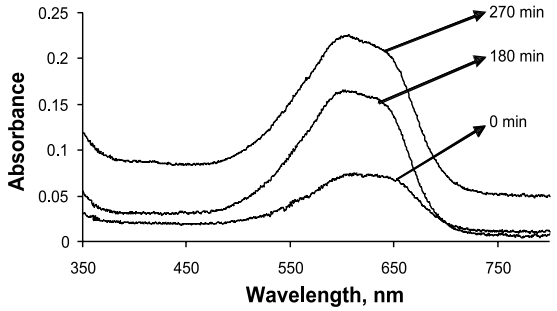
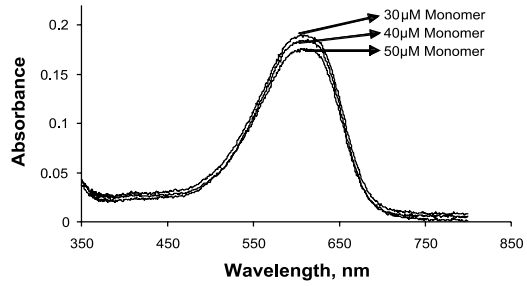


Fig. 4. Absorption Spectrum for Fibril Extension Studies

50 μM β_2m fibrils incubated for 270 min were observed for absorbance changes with 60 μM of TB. There is a marked increase in the absorbance measurements at 606.50 nm, taken as the average λ_{max}, of β_2m fibrils incubated at 37°C through time.

(a)



(b)

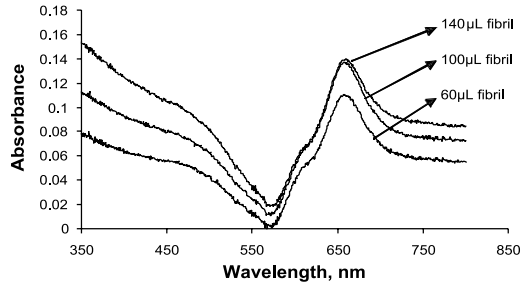


Fig. 5. Absorption Spectra of (a) β_2m Monomer with 60 μM TB and (b) β_2m Fibrils with 60 μM TB

The absorption spectra shown above represents the reaction of different amounts of β_2m fibrils and β_2m monomers added to a fixed concentration of TB. Addition of 30–50 μM of β_2m monomer to 60 μM of TB exhibited negligible effect on the absorbance, and no shift in the maximum absorption wavelength. On the other hand, a slight increase in the absorbance measurements and a spectral shift was observed when 60–140 μl of β_2m fibrils were added to 60 μM of TB in solution.

tion (Fig. 2). A short lag phase was observed before the fluorescence intensity increased steadily. This suggests that the fluorescence induced by the binding of ThT to β_2m fibrils is a good indicator of fibril formation. As such, the response of ThT to β_2m fibril formation serves as a good gauge to measure the ability of TB to monitor β_2m fibrillogenesis.

TB Binding

The assay developed is an easy detection tool for the presence of β_2m fibrils spectrophotometrically using the TB dye. To determine if the method specifically binds β_2m fibrils, we compared assay results from β_2m monomer with β_2m fibrils.

Significant shifts in the maximum absorption wavelength were observed for the TB + β_2m fibril solutions as compared to negligible changes in the λ_{max} of the TB + β_2m monomer solutions (Fig. 3). This is an indication that TB certainly binds to the fibrils. The similarity in structure of TB with the commonly used amyloid dye, Congo red, may explain the affinity of the dye to the β-sheet structure of the fibril. The presence of hydrogen-binding sites[9] and the symmetrical feature of TB most likely contribute to the observed specific binding. Apparent also is the shouldering of the absorption peaks for the TB + β_2m fibril samples. This may suggest binding of the dye at different sites of the fibril due to a change in conformation from the native monomeric state to a more complex fibrillar state.

Fibril extension studies were done to verify the binding of TB to β_2m fibrils. A fixed concentration of β_2m fibrils were incubated at increasing time periods. Comparable to the ThT assay, absorbance measurements were observed to increase with longer incubation of β_2m fibrils. This kinetic study demonstrates that TB can indeed monitor fibril formation.

To further validate the binding of TB to β_2m fibril, the effect of various amounts of β_2m monomer and fibril was determined against a fixed concentration of the dye. Figure 5 illustrates a pattern that at constant incubation time of the fibrils and at a fixed concentration of TB, an increase in the amount of fibrils results to an increase in absorbance. Evidently, TB particularly binds to β_2m fibrils.

In conclusion, TB has been found to bind specifically with the fibrils of β_2m. This was demonstrated by a red shift in the maximum absorption wavelength through UV-Vis spectroscopy. Like ThT, a dye used in standard assays to determine the extent of fibril formation of proteins, TB may likewise be used to assay other proteins having the propen-

sity to form fibrils. These proteins include the Aβ peptide of Alzheimer's disease, the prion protein of transmissible spongiform encephalopathy, and the α-synuclein of Parkinson's disease. This simple method of using TB in conjunction with UV-Vis spectroscopy for amyloid fibril elucidation may further be developed with the use of other staining dyes.

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