

Aggregation and fibrillation of bovine serum albumin

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Abstract

The all- α helix multi-domain protein bovine serum albumin (BSA) aggregates at elevated temperatures. Here we show that these thermal aggregates have amyloid properties. They bind the fibril-specific dyes Thioflavin T and Congo Red, show elongated although somewhat worm-like morphology and characteristic amyloid X-ray fiber diffraction peaks. Fibrillation occurs over minutes to hours without a lag phase, is independent of seeding and shows only moderate concentration dependence, suggesting intramolecular aggregation nuclei. Nevertheless, multi-exponential increases in dye-binding signal and changes in morphology suggest the existence of different aggregate species. Although β -sheet content increases from 0 to ca. 40% upon aggregation, the aggregates retain significant amounts of α -helix structure, and lack a protease-resistant core. Thus BSA is able to form well-ordered β -sheet rich aggregates which nevertheless do not possess the same structural rigidity as classical fibrils. The aggregates do not permeabilize synthetic membranes and are not cytotoxic. The ease with which a multidomain all- α helix protein can form higher-order β -sheet structure, while retaining significant amounts of α -helix, highlights the universality of the fibrillation mechanism. However, the presence of non- β -sheet structure may influence the final fibrillar structure and could be a key component in aggregated BSA's lack of cytotoxicity.

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1. Introduction

Under various conditions, many proteins can aggregate to regular arrays of β -sheet rich filaments or fibres of indefinite length, often coiled together in higher-order structures [1,2]. The ability to fibrillate is independent of the original native structure of the protein, whose amino acid sequence primarily appears to play a role in terms of filament arrangement [3], fibrillation kinetics [4] and overall yield and stability of the fibrils [5,6]. It typically occurs under conditions that stabilize partially folded or unfolded states [7]. For example, the all- α protein myoglobin

fibrillates under conditions (pH 6.5, 65 °C, heme factor removed) where the denatured state dominates [8,9]. Although fibrillation is associated with the development of neurodegenerative diseases, cytotoxicity appears to be associated with pre-fibrillar aggregated states, either because of their ability to permeabilize cell membranes to general ion flux [10–12] or because the rather diffuse hydrophobic surface may catalyze unwanted reactions [13,14]. The fibrils themselves appear to be relatively innocuous, except when they associate to very high levels in e.g. systemic amyloidoses, causing organ failure [15]. In addition, fibrils are associated with beneficial functions such as the formation of bacterial biofilm that allows microbial organisms to adhere to cell surfaces [16].

A typical fibrillation time profile, usually monitored with the help of the fibril-specific dyes Thioflavin T or Congo Red, commences with a long lag time followed by a relatively short

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elongation phase where fibrils accumulate [17]. The length of the lag time is thought to be determined by the formation of the fibrillation nucleus, a thermodynamically and kinetically unfavourable state that functions as a template for fibril growth. In such cases, addition of sonicated fibrils (which are assumed to be equivalent to nuclei) can reduce or abolish the lag phase [17]. However, other models including fragmentation, branching and heterogeneous nucleation can also give rise to the characteristic sigmoid appearance [18]. Some proteins, such as acyl phosphatase, commence aggregation without a lag phases in the rise of the ThT signal [19]; however, in this case the first aggregate species to accumulate are relatively amorphous and *bona fide* fibrils only form much later [20].

Here we describe fibrillation of the ubiquitous bovine serum albumin (BSA). BSA is a 583-residue protein consisting of three homologous all- α domains, organized in a heart-shaped structure [21]. It constitutes ca. 60% of all plasma protein and binds and transports a large number of physiological and non-physiological ligands [22]. Although its aggregation properties have been studied for a number of years, we are not aware of any reports of actual fibrillation. BSA contains 17 disulfide bonds and one unpaired cysteine (Cys34), which facilitates dimerization and also influences higher-order association, since the rate of aggregation is slowed down if Cys34 is covalently bound to another compound [23]. Aggregation of 1 g/l BSA at elevated temperatures proceeds through the formation of an intermediate with an increased content of β -sheets [24]; irreversible intermolecular β -sheet formation only occurs at 70 °C and above [25]. Secondary structural changes are essentially complete within the first 70 min of incubation at 70 °C, whereas tertiary changes occur over longer time scales [23]. In view of the fact that aggregation requires access to non-native states, it is not unexpected that aggregation is inhibited by the addition of ligands such as Tween 80, tryptophan and caprylate which stabilize the native state.

We show that BSA forms aggregates with amyloid characteristics within minutes upon incubation at neutral pH at elevated temperatures. The reaction proceeds without a lag phase, is only linearly dependent on protein concentration and is not accelerated by seeding, nor do the ensuing aggregates show any cytotoxicity. The ease with which BSA fibrillates highlights once again the ubiquitous nature of fibrillation, which does not exempt even multi-domain all- α proteins; at the same time it serves to illustrate that fibrils can tolerate a high amount of non- β structure, presumably due to their ability to relegate α -helix structure to parts outside the fibrillar core.

2. Materials and methods

2.1. Materials

Bovine Serum Albumin fraction V (BSA), Congo Red (CR) and Thioflavin T (ThT) were from Sigma-Aldrich (St. Louis, MO). Trypsin was from Promega (Madison, WI). 1,2-Dioleoyl-*sn*-Glycero-3-phosphocholine (DOPC) and the sodium salt of 1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)] (DOPG) were from Avanti Polar Lipids (Alabaster, AL). Calcein (sodium salt) was from Merck (Darmstadt, Germany). All chemicals were of the highest purity available. The buffer used was in all cases 20 mM Tris–HCl pH 7.4 unless otherwise stated.

2.2. Preparation of BSA aggregates

BSA was dissolved in buffer to a final concentration of typically 1–5 mg/ml as indicated in the text. Before incubation the solution was filtered through a 0.2- μ m filter into sterile test tubes. The samples were incubated at a specified temperature (60–75 °C) in a water bath without agitation. Samples were taken out at intervals and stored on ice before adding CR or ThT. For longer storage before measurement, tubes with samples were removed and frozen at –20 °C at 0 h, 2 h and 96 h.

2.3. Thioflavin T spectroscopy

Typically, 5 μ l BSA sample was mixed with ThT to a final concentration of 20 μ M ThT in buffer. Fluorescence was measured in an LS55 fluorimeter (Perkin Elmer, Wellesley, MA) with excitation and emission wavelengths of 450 and 482 nm, respectively.

2.4. Protease digestion experiments

2.5 g/l BSA was incubated at 70 °C in buffer for 4 days, after which they were diluted out to 0.25 g/l in a 96-well plate in the presence of 40 μ M ThT and 1% trypsin (w/w). Recordings were started within a few minutes on a SpectraMax GeminiXS plate reader (Molecular Devices, Sunnyvale CA). Excitation was at 450 nm with emission at 485 nm at 37 °C. Recordings were carried out every 20 min, preceded by 5 s of shaking. Between measurements, the plate was shaken for 180 s.

2.5. X-ray fiber diffraction

Fibrils were formed by incubating a 2-mg/ml BSA solution in Tris buffer at 70 °C for 4 days. The solution was centrifuged at 32,000 $\times$ g for 30 min and drops of sample from the bottom of the Eppendorf tube were placed between two end-to-end glass pipettes sealed with wax. After several days at 5 °C, one drop had turned into a thin, gel-like fibre. Diffraction data were collected at beamline I911-3, MAX-lab, Lund, Sweden, at 1.00 Å. During the 6-min exposure, the fibre was rotated 1°. Diffraction intensity, in general, was low in accordance with the gel-like nature of the fibre. Intensity profiles were made by integrating from 30 to 150° and between 150 and 240°. The intensity profiles show a sharp peak at 4.65 Å and several peaks at 9.7–10.8 Å. The fact that several sidechain peaks are present could indicate that several fibril morphologies are present in solution.

2.6. Calcein experiments

Vesicles containing 80% DOPC and 20% DOPG by weight were prepared essentially as described [26]. Briefly, after dissolution in methanol and evaporation of methanol, the lipid mixture was resuspended to 12.5 mg/ml in buffer containing 40 mM calcein, subjected to freeze–thawing and extruded through a 100-nm filter. Free calcein was removed by desalting over a pre-equilibrated PD10 column, leading to an approximately 2-fold dilution in the most concentrated eluted fraction of vesicles. The vesicle solution was then diluted out an additional 100-fold in buffer in the presence of 0.22 mg/ml BSA. The BSA was diluted from a 2.5-mg/ml solution which had been incubated in buffer at 70 °C for 0–120 min. At 40 mM inside the vesicles, calcein quenches its own fluorescence, and fluorescence will increase if it is diluted by exiting the vesicle [27]. As positive control we used the antimicrobial peptide Novispirin [26], where we observed up to 90% leakage compared to full vesicle rupture upon addition of 2% Triton X-100. The fluorescence emission at 515 nm was recorded every 4 s in the LS55 fluorimeter using excitation at 490 nm and slit widths of 2.5 nm for both excitation and emission.

2.7. Congo Red absorption

50 μ l sample was mixed with CR in PBS-buffer (140 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄ pH 7.4) to a final CR concentration of 20 μ M and a total volume of 1 ml. The absorbancies at 403 and 541 nm were

measured using a 1-cm path length cell. The concentration of fibrils bound to CR can be calculated using the following relationship [28]:

$$[\text{Fibril}](\text{mM}) = A_{541}/47.8 + A_{403}/38.1 \quad (1)$$

2.8. CD spectroscopy

Far-UV CD wavelength spectra and temperature scans were recorded on a Jasco J-810 spectropolarimeter (Jasco Spectroscopic Co. Ltd., Hachioji City, Japan) equipped with a Jasco PTC-348WI temperature control unit using a 1-mm quartz cuvette. 0.25 mg/ml BSA in 20 mM Tris pH 7.4 was used. Thermal scans were recorded at 220 nm with a scan-rate of 60 °C/h and analyzed as described [29] to obtain the melting temperature T_m .

2.9. Electron microscopy

Electron microscopy was carried out as described [30]. Briefly, a 5- μ l aliquot of BSA was placed on a 400-mesh, carbon-coated, glow-discharged grid for 30 s. Grids were washed in two drops of double-distilled water and stained with 1% (w/v) phosphotungstic acid (pH 6.8) and blotted dry on filter paper. Samples were viewed with a Jeol 1010 transmission electron microscope.

2.10. Fourier Transform Infrared Spectroscopy

Around 10 μ l of 2.5 mg/ml BSA solution was loaded onto the crystal of a TENSOR 27 spectrometer (Bruker) with a Specac Golden Gate single-reflection ATR unit (Specac Ltd., Orpington, UK) until the intensity of the amide I band reached 0.15–0.20 absorbance units. The sample was dried with nitrogen until the water signal had stabilized, and afterwards an absorption spectrum was recorded in the range of 1000–4000 cm^{-1} . Background measurements were based on 64 scans, and sample measurements were based on 32 scans. The data obtained by FT-IR were processed using the options of OPUS version 5.5. To reduce noise, each spectrum was smoothed by application of the Savitsky–Golay algorithm employing 13 smoothing points. In order to enhance the resolution, the smoothed spectra were deconvolved using Fourier self-deconvolution by application of a Lorentzian line shape and a noise reduction factor of 0.5. The deconvolution factor was determined individually for each spectrum by increasing the value until the point of oscillation. Linear baseline correction of the amide I band was performed for each spectrum by manually adding a tangent at $\sim 1700 \text{ cm}^{-1}$ and a baseline point at the absorbance minimum between the amide I and amide II bands. To separate possible overlapping bands in the spectra, the curve fitting option was applied. The frequency range of the fit was chosen to be the local minimum surrounding the amide I band (~ 1710 – 1580 cm^{-1}). By application of the Levenberg–Marquardt algorithm, which uses iteration, each spectrum was fitted to the following four peaks: 1620 cm^{-1} (β -sheets), 1640 cm^{-1} (unordered structures), 1656 cm^{-1} (α -helices) and 1672 cm^{-1} (β -turns). Iteration was performed to improve the root mean square (RMS) noise parameter, until the smallest possible RMS-value was obtained [31]. The area constituted by each of the four peaks of the fit was determined by the rectangular integration method and was used to estimate the secondary structure content of aggregated BSA.

2.11. Dynamic light scattering

Hydrodynamic radius R_h was measured using a PN3000 batch DLS particle size detector (Postnova Analytics, Landsberg, Germany) at 25 °C, using a viscosity of 0.0089 poises, a refractive index of 1.333 and a sampling time of 3 ms. Prior to each measurement, buffer was measured to check that the cuvette was properly cleaned.

2.12. Growth of neuroblastoma cells in the presence of aggregated BSA

Samples were prepared in 96-well microplates and all wells without samples were filled with 200 μ l PBS to minimize evaporation. To each well was loaded 100 μ l SH-SY5Y growth medium with or without 10.000 SH-SY5Y

neuroblastoma cells (ATCC number CLR-2266, LGC Promochem, Teddington, UK). The assay was started by adding 25 μ l buffer, with or without 5 mg/ml sonicated BSA previously incubated for 0, 2 or 96 h at 70 °C (giving a final BSA concentration of 1 mg/ml). All sample combinations were loaded in triplets, and for each of the six BSA combinations a corresponding triplet was loaded without cells, to be used as blank. One triplet was also loaded without both cells and BSA and one only without BSA.

2.13. MTT assay for fibril cytotoxicity

The Vybrant® MTT Proliferation Assay (Invitrogen, Carlsbad, CA) was used. MTT stock solutions were prepared as described by the manufacturer. The setup of the assay deviates somewhat from the Vybrant MTT protocol because our assay needs to measure the change in viability over a period of approximately 64 h. Therefore ten 96-well microplates with various samples were prepared and incubated simultaneously. One plate was then taken out and measured for each time point, and because the assay is destructive each plate was then removed.

The procedure for each measurement was performed as described in the Vybrant® MTT protocol, with the exception that DMSO was used to solubilise the cells and crystals as described by the Quick Protocol Option in the Vybrant® MTT protocol. The absorbance of the solubilised formazan crystals was measured at 560 nm using a Victor Wallac 1430 plate reader spectrophotometer. The viability of the treated cells is normalized with regard to the viability of untreated cells incubated for the same time period.

3. Results

3.1. Formation of ThT- and CR-binding aggregates of BSA

According to our CD thermal scans which monitor ellipticity at 220 nm as a function of temperature, BSA denatures with a midpoint melting temperature of $65.1 \pm 0.35 \text{ °C}$ at pH 7.4 (data not shown). We reasoned that aggregation and fibrillation of BSA required the protein to access the denatured state, and therefore incubated BSA at temperatures around and above the melting temperature, following the process with the two fibril-binding dyes ThT and CR. While incubation at 70 °C over extended periods of time does not lead to any visible turbidity, there is a strong increase in ThT fluorescence (Fig. 1A) and an increase in CR absorption which is accompanied by a small redshift from 495 to 500 nm in the absorption spectrum (Fig. 1B). The absorption of BSA alone after incubation at 70 °C only contributes ca. 20% of the increase in CR absorption (Fig. 1B), indicating that the change is mainly caused by the formation of Congo Red-binding species, although the red shift is not as pronounced as seen for classical fibrils [28]. Over a time scale of a few hours, the signal change rises to a plateau level for both dyes (Fig. 1C and D). This development can be fitted to a single exponential decay without any lag phase, leading to an apparent half-life of aggregation $t_{1/2}^{\text{agg}}$. To characterize this process in more detail, we examined its temperature dependence. Our study is limited to the region 60–75 °C; below 60 °C (65 °C in the case of CR) no aggregation is observed, while aggregation above 75 °C is too fast to be measured reliably according to our procedures. An Arrhenius plot of $t_{1/2}^{\text{agg}}$ shows a strong linear correlation with $1/T$ (Fig. 2A) for both dyes. However, the rate constants, while similar in magnitude, are not identical for the two dyes, giving rise to a substantial difference in activation energy, E_a . As determined by the CR assay, E_a is $72.7 \pm 3.0 \text{ kcal mol}^{-1}$, while ThT gives rise to a significantly lower E_a of $24.6 \pm$

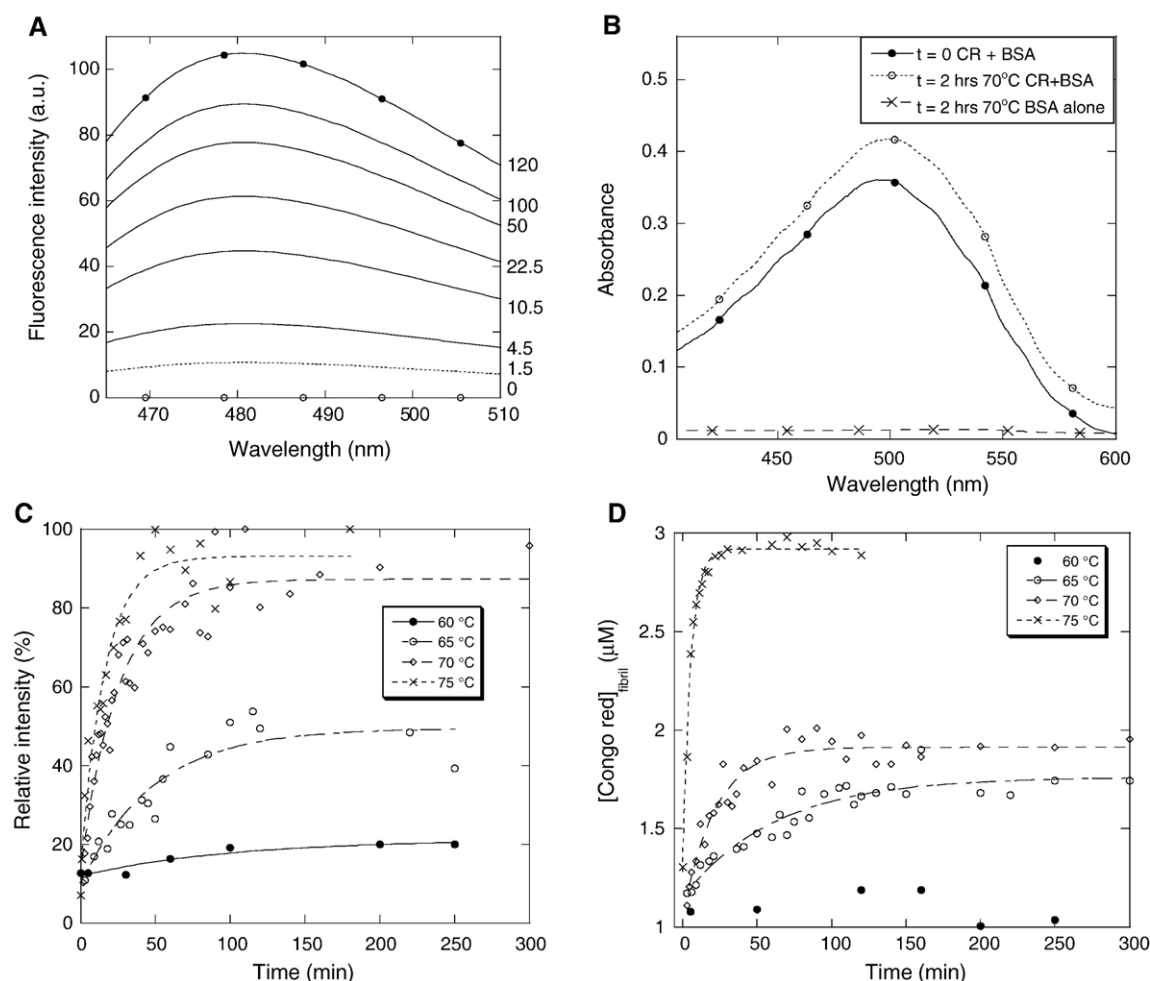


Fig. 1. (A) Aggregation of BSA leads to a marked increase in ThT fluorescence. 2.5 mg/ml BSA was incubated at 70 °C and pH 7.4, and at the indicated timepoints (in min) aliquots were removed, diluted 24-fold and incubated with 40 μM ThT, after which fluorescence spectra were recorded. The change in emission intensity as a function of time is also shown in panel C. (B) Absorption spectrum of CR under same conditions as in panel A. For clarity, only time points 0 and 120 min are shown. The spectrum of BSA alone after 2 h at 70 °C is included to show that this spectrum alone cannot account for more than a small fraction of the increase in Congo Red absorption. (C) ThT fluorescence over time measured at 482 nm for 2.5 mg/ml BSA incubated at 60–75 °C. Fluorescence is given as a percentage of the highest fluorescence intensity obtained. For 60, 65 and 70 °C, the maximum value of 70 °C is applied, and for 75 °C the maximum value of 75 °C is applied. (D) The concentration of CR bound to 2.5 mg/ml BSA incubated at 60–75 °C for different periods of time. Data in panels B and C are fitted to a single exponential function to obtain the half-life of aggregation $t_{1/2}^{\text{agg}}$.

1.7 kcal mol⁻¹. This, together with the lack of a CR signal at 60 °C, suggests that the two dyes are monitoring different aspects of the aggregation process.

This is substantiated by a time-study stretching over several days, which reveals that the CR-monitored process is completed within a few hours, while a second and much slower relaxation phase with a half-life of around 25 h is observed by ThT fluorescence (Fig. 2B). Electron microscopy revealed significant formation of stumpy aggregates already after 2 h (Fig. 3A). Over the next 4 days, curly fibrillar structures with a diameter around 10 nm emerge (Fig. 3B). The most reliable demonstration of fibril-like structure is provided by X-ray fiber diffraction data, which directly report on the molecular structure. Classical fibrils show typical diffraction patterns at around 4.5–5 Å (corresponding to inter-strand distances) and 9.5–11 Å (inter-sheet distances) [32]. We managed to prepare a thin gel-like fibre, which led to a diffraction pattern with low intensity (Fig. 3C) but

nevertheless contained a clearly discernible diffraction ring around 4.65 Å as well as several peaks around 9.7, 10.4 and 10.6 Å (Fig. 3D). The fact that several inter-sheet peaks are present could indicate that several fibril morphologies are present in solution.

DLS data also suggested growth of aggregates, since the average hydrodynamic radius grew from 2 to 6 nm within the first 30 min of incubation at 70 °C (Fig. 2B insert). After 30 min, the particles were too large to allow accurate size determination, although they remained soluble since they could not be spun down.

3.2. Fibrillation is independent of seeding and shows linear concentration dependence

Typical fibrillation processes involve a lag phase followed by a relatively rapid elongation phase, which stabilizes at a plateau

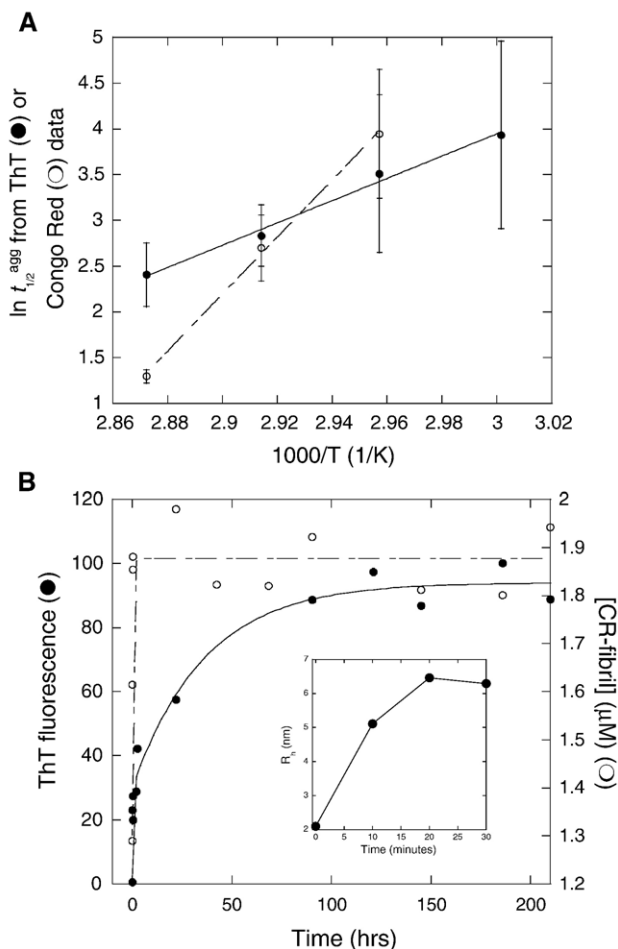


Fig. 2. (A) $\ln t_{1/2}^{agg}$ (2.5 mg/ml BSA) plotted versus the reciprocal of the aggregation temperature. Error bars indicate errors from fits in Fig. 1. The linear fits yield slopes of $12.2 \pm 0.8 \times 10^3$ (ThT) and $31.2 \pm 1.3 \times 10^3$ (CR) K^{-1} , leading to activation energies E_a of 24.6 ± 1.7 (ThT) and 72.7 ± 3.0 (CR) $kcal\ mol^{-1}$, respectively. (B) Development of ThT and CR signal over 210 h for BSA incubated at 2.5 mg/ml at 70 °C. The CR signal has been fitted to a single exponential and the ThT signal to a double exponential (ThT half-lives 8.2 ± 5.0 and 1505 ± 512 min, respectively). Insert: change in R_h over time when 2.5 mg/ml BSA in buffer is incubated at 70 °C.

level when all monomers have been incorporated into fibrils [17]. The absence of a lag phase would however suggest that the nucleus is either formed very rapidly (in the dead time of mixing) or the aggregation process we monitor is not classical nucleation-based fibrillation. If the nucleus consists of more than one molecule, nucleus formation will be slowed down at lower BSA concentrations. However, when aggregation of BSA was monitored at concentrations of 0.5–10 mg/ml, we see no apparent lag phase at low concentrations (Fig. 4A) and only observed a modest linear dependence of aggregation rate on protein concentration (Fig. 4B). The degree of aggregation monitored by ThT shows an excellent proportionality with protein concentration (Fig. 3B), indicating that aggregation goes to completion at all concentrations. Additionally, we investigated the effect of seeding the aggregation process with pre-formed aggregates (formed by incubating BSA for several hours at a given temperature, followed by sonication to disperse the aggregates and expose growing ends to monomers). However,

there was no significant acceleration of aggregation at 60–70 °C (data not shown). At 55 °C, aggregation does not occur in the absence of seeding, and the addition of seeds did not change this (data not shown).

The aggregation process did not appear to be mediated by covalent intermolecular disulfide links mediated by BSA's single unpaired Cys34 residue, since SDS-PAGE gels run under in the absence of reducing agents do not show substantial increases in dimers or other higher-order aggregates (data not shown). Arakawa and Kita (2000) have observed loss of monomeric BSA and formation of multiple aggregated bands by native-PAGE after incubation of BSA at 60 °C for 15 min. In view of our results, we suggest that these aggregates are linked by non-covalent interactions.

3.3. Fibrils are readily degraded by protease according to both SDS-PAGE and ThT fluorescence

In order to identify a possible protease-resistant structurally robust core within the BSA aggregate, we incubated 4-day old BSA aggregates with 1% trypsin (w/w). This should be an appropriate protease concentration for limited proteolysis; for comparison, lysozyme fibrils were only partially degraded upon incubation with 3% (w/w) pepsin [33]. We monitored the process by SDS-PAGE in parallel with ThT fluorescence. However, both techniques show complete degradation of the aggregates (Fig. 5 insert); the BSA band disappeared completely on the SDS gel and was not replaced by any well-defined lower molecular weight band (data not shown), while the ThT fluorescence declined to background base level according to a simple exponential decay ($t_{1/2}$ 9.3 ± 0.5 h).

3.4. Aggregation is accompanied by an increase in β -sheet structure and is inhibited by intermediate concentrations of TFE

To investigate the nature of the conformational changes associated with aggregation, we recorded FTIR spectra of BSA aggregates over time. Native BSA is approximately 70% α -helical and contains no β -sheet structure, as confirmed by FTIR (Fig. 6A). Within a few minutes of incubation at 70 °C, however, there was a clear decrease in α -helicity and a corresponding increase in β -sheet content, and this level stayed constant over a period of several days (Fig. 6B and C), confirming previous observations [23–25]. Thus both CR and ThT monitor the rapid rise in β -sheet-rich aggregates occurring within a few minutes; the additional rearrangements monitored by ThT over a period of several days do not involve significant increases in β -sheet structure, but take place in the presence of significant amounts of α -helical structure.

Our data suggest that a prerequisite for aggregation is that BSA can assume a non-native conformation. This is further substantiated by the observation that 5 mM octanoic acid, which raises the melting temperature of BSA from 65.1 to 83.1 ± 0.42 °C, completely abolishes BSA aggregation at 70 °C (data not shown). However, the nature of the denatured state also plays a role. The denaturing alcohol trifluoroethanol (TFE) generally

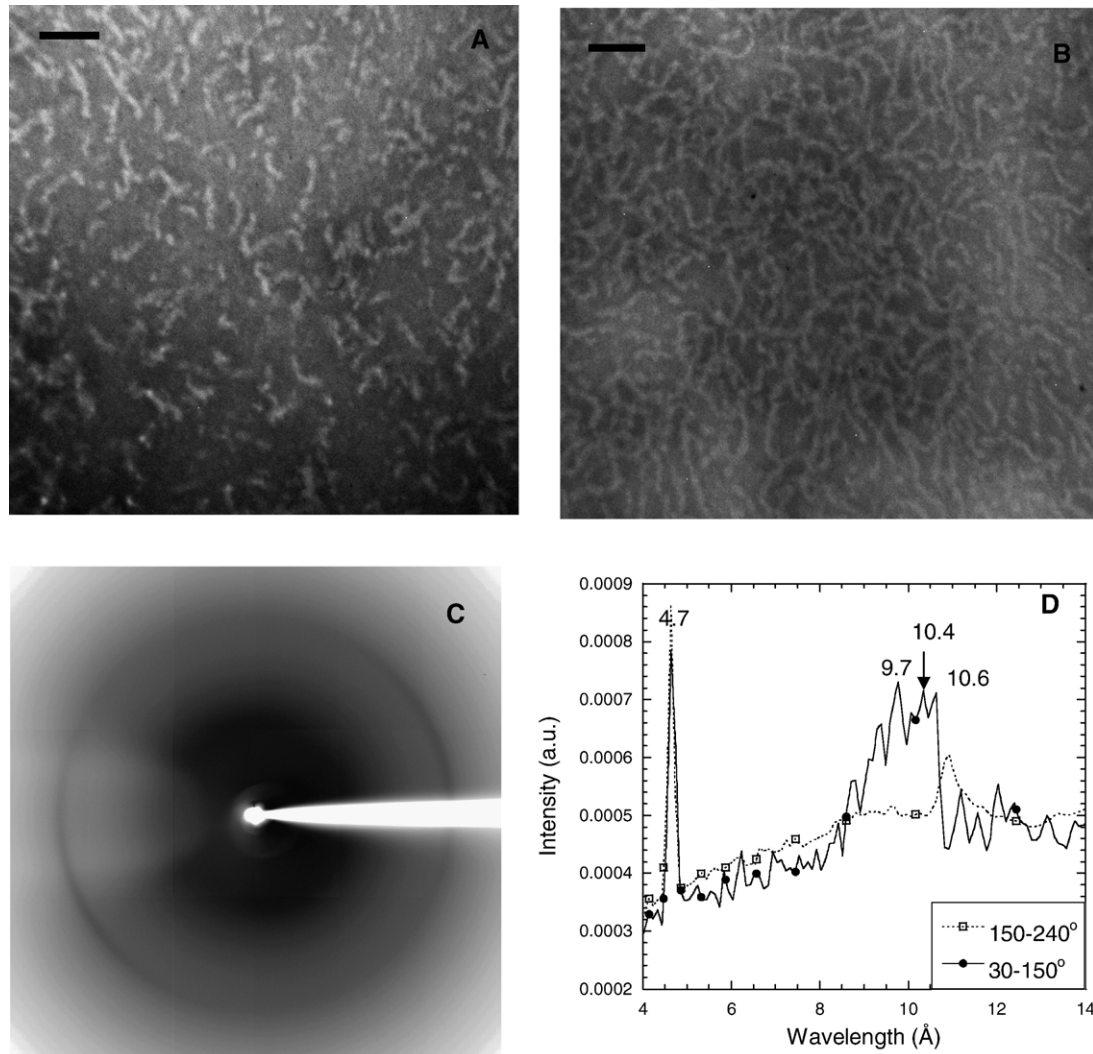


Fig. 3. Electron microscopy images of 2.5 mg/ml BSA incubated at 70 °C for (A) 2 and (B) 96 h. The black distance bar indicates 100 nm. (C) X-ray fiber diffraction pattern of a BSA fiber. (D) Intensity profiles integrated from 30 to 150° (containing the black inner circle) and between 150 and 240° (where the black circle is absent).

induces an increase in α -helicity in proteins at high concentrations, but intermediate concentrations (around 25–30% for acyl phosphatase [34]) promote formation of β -sheet structures, induce extensive precipitation of membrane proteins [35] and can stimulate aggregation and fibrillation. We therefore investigated the consequences of incubating BSA with TFE. Incubation of BSA at pH 7.4 with 0–80% TFE at room temperature over several days led to a small increase in ThT signal with a clear optimum around 30% TFE (Fig. 7A), similar to the conditions in [34]. However, the total amount of aggregation was less than 20% of thermally aggregated BSA and was too small to be detected clearly by FTIR.

TFE destabilizes BSA, leading to a decrease in T_m to 53.0 ± 0.15 °C in 10% TFE and a lack of a cooperative transition in 20% TFE (data not shown). This destabilization does not encourage the aggregation process. When BSA is incubated with 0–20% TFE at 70 °C, the aggregation half-time is unchanged in 10% TFE (8.9 ± 1.0 min compared to 10.6 ± 1.4 min in 0% TFE), and slows dramatically to 154 ± 22 min in 20% TFE (Fig. 7B).

3.5. MTT assay for fibril cytotoxicity shows BSA is beneficial for cell growth irrespective of its aggregated state. Lack of cytotoxicity is confirmed by membrane permeabilization assays

We next investigated the potential cytotoxicity of the BSA aggregates by incubating aggregates with the neuroblastoma cell line SHSY5Y and measuring cell viability using the MTT assay, in which MTT is metabolized to insoluble dark blue formazan by the mitochondrial respiratory chain of healthy cells [36]. The initial light scattering experiment showed that the fibrils, while non-turbid, contributed a significant amount of light scattering to the absorbance measurements. Therefore two sets of samples were included, with and without sonication. The sonicated samples showed a lower light scattering, but a consequence of the sonication could be an altered bioactivity and therefore both sets were included in the assay.

The general tendency is that there is an initial steady phase of around 25 h, where there is no significant change between the viability of treated and untreated cells (Fig. 8). In the subsequent

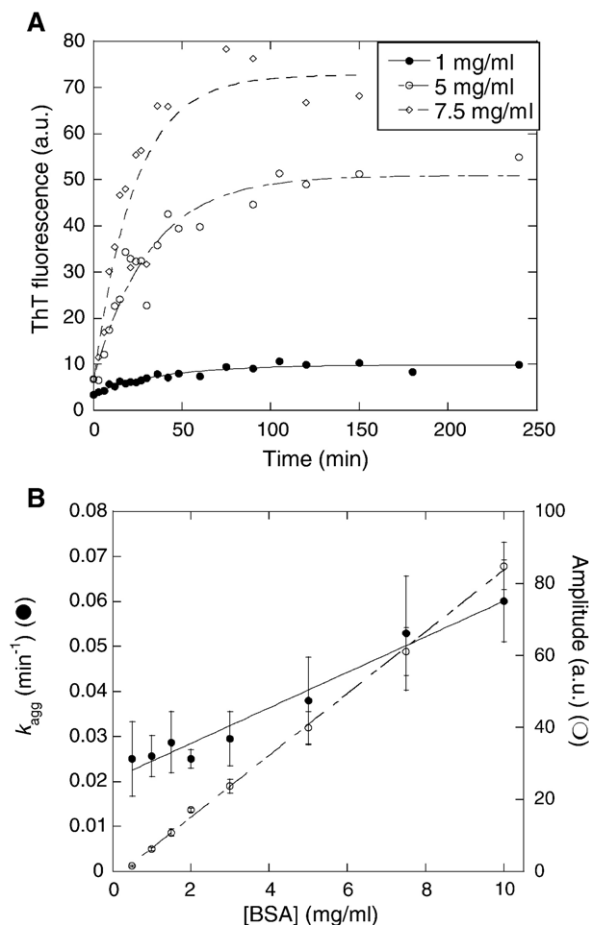


Fig. 4. (A) Representative time profiles for development of ThT signal when BSA is incubated at different concentrations at 70 °C. Data are fitted to a single exponential function to obtain the half-life of aggregation $t_{1/2}^{agg}$ and the associated signal change (amplitude). (B) $t_{1/2}^{agg}$ and amplitude as a function of BSA concentration. Error bars indicate errors from fit in panel A.

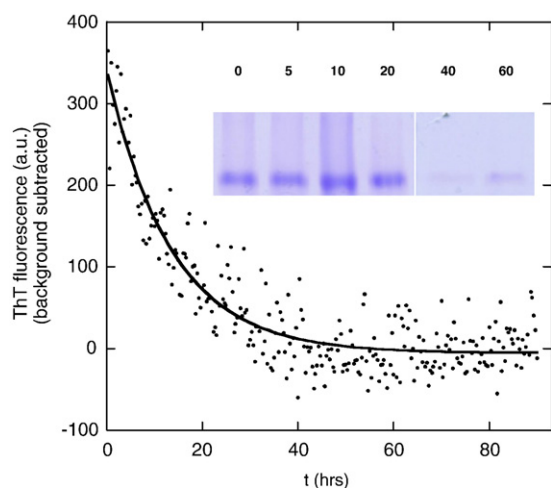


Fig. 5. Decline in ThT fluorescence to background levels when BSA aggregates formed by incubation for 4 1/2 days at 70 °C are incubated at 0.25 mg/ml with 1% trypsin (w/w). Background level (ThT in presence of 1% trypsin) subtracted. Insert: disappearance of BSA band upon incubation with trypsin. No lower-molecular weight appears (data not shown), indicating that the aggregates are completely degraded.

40 h, an increase in the viability of the treated cells of 300–400% is observed, indicating that the cells incubated with BSA achieve a higher viability than the untreated cells. The viability difference disappears towards the end of the experiment, possibly because the BSA has become metabolized in the

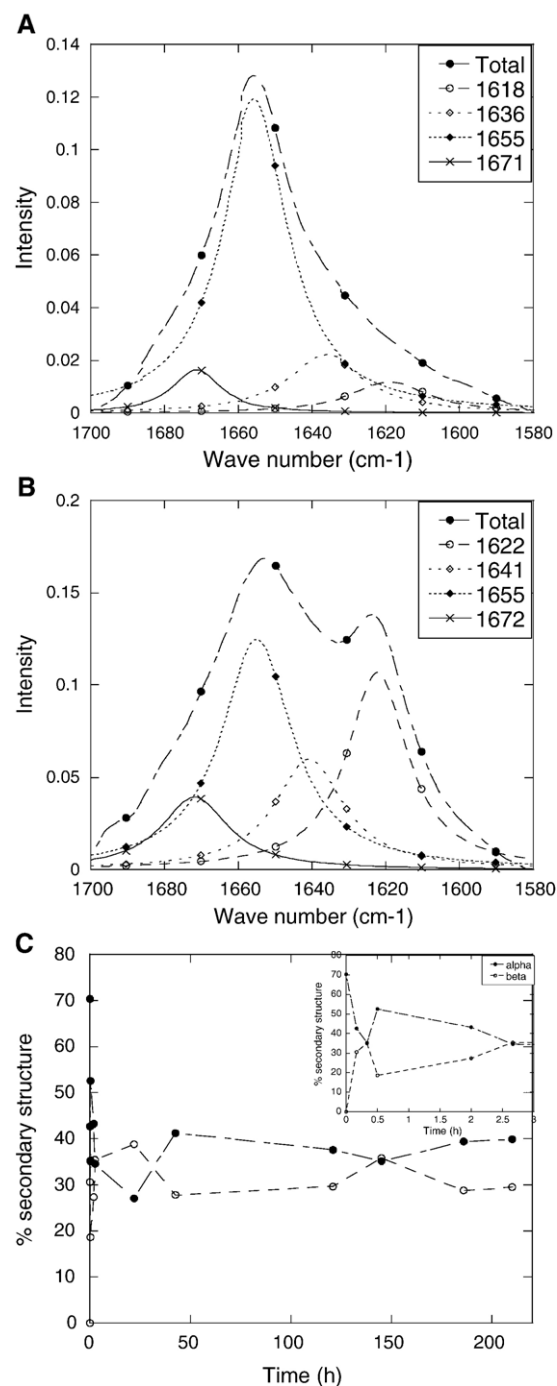


Fig. 6. FTIR spectra of 2.5 mg/ml BSA incubated at 70 °C after (A) 0 h and (B) 210 h. The total spectrum has been deconvoluted to 4 principal components representing different elements of secondary structure based on peaks at different wavelengths (see Materials and methods). (C) Development of β -sheet structure over time upon incubation. Insert shows development over the first 3 h.

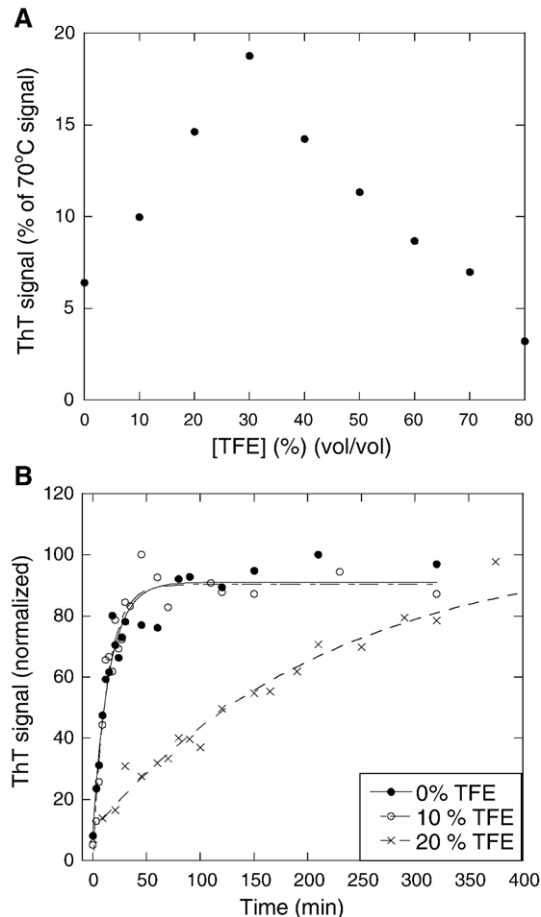


Fig. 7. (A) ThT signal of 2.5 mg/ml BSA incubated 2 days at room temperature with 0–80% TFE (background subtracted; sample diluted 24-fold for ThT fluorescence). (B) Time-course of ThT signal upon incubation of 2.5 mg/ml BSA with 0–20% TFE at 70 °C.

meantime. However, the change in viability is not dependent on the aggregated state of BSA, since non-incubated BSA has the same effect as the aggregated samples.

A priori this increased viability might result from the increased amount of proteins in the wells (given the relative ease with which BSA aggregates can be degraded by proteases), but a nutritional effect is unlikely, as the SH-SY5Y medium already includes an excess of amino acids and proteins. In addition, the cell density in the wells is too low to require additional amino acids for the duration of the experiment. Given that unaggregated BSA has the same effect, the improved viability is unlikely to arise from the fibrils providing additional attachment sites for the cells can attach. Therefore, though it may have some practical uses, we are at present unable to explain the beneficial effect of BSA.

Aggregate cytotoxicity has been suggested to be connected with the ability to permeabilize cell membranes [11,37,38]. However, BSA aggregated by exposure to 70 °C between 0 and 120 min does not show any ability to liberate calcein from their entrapment inside vesicles containing 80% DOPC and 20% DOPG (data not shown). This is consistent with the lack of cytotoxicity towards SHSY5Y cells.

4. Discussion

The overall aim of this study was to test the generic ability of proteins to aggregate to β -sheet rich fibrillar structures using the ubiquitous and all α -helical “protector of the plasma” BSA as a test case. The “generic amyloid” hypothesis essentially states that the ability of proteins to form ordered fibrillar cross- β structures is inextricably linked to the nature of the protein backbone [39,40] and is independent of the structure of the native state. Thus, while some proteins obviously fibrillate more readily than others, it should be possible to find conditions that are sufficiently destabilizing for the native state to allow the protein to explore alternative conformations and lock on to the stable β -sheet aggregated state. A precedent for fibrillating all- α helix proteins is provided by apomyoglobin [8], but this is a small single domain protein and we find it of interest to explore whether this feature can be observed for a large multi-domain protein such as BSA, where an abundance of α -helical structure might impede the accessibility of isolated β -sheet regions.

4.1. Non-classical amyloid structures may be related to a preponderance of α -helix structure

We observe that simple incubation at physiological pH but at elevated temperature is sufficient to induce aggregates, which test positive for amyloid according to two standard assays, namely ThT fluorescence and X-ray fiber diffraction. While the high temperatures required for aggregation renders the process physiologically unrealistic, the readiness to aggregate supports the generic amyloid hypothesis. Nevertheless, the aggregates formed in this manner show several properties which deviate from classical fibrils.

Firstly, CR shows a rather modest red-shift in its absorption maximum (from around 495 to 500 nm) compared to e.g. A β

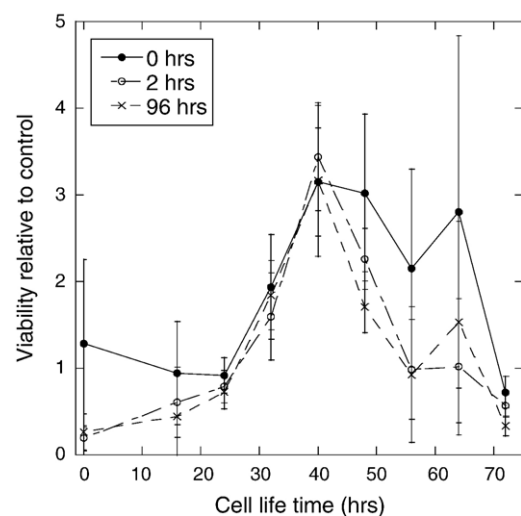


Fig. 8. Cell viability of SHSY5Y cells incubated in the presence of 1 mg/ml BSA (native or aggregated for 2 or 96 h at 70 °C in buffer). Viability determined using the MTT assay. The viability of the treated cells is normalized with regard to the viability of untreated cells incubated for the same time period.

fibrils, where peaks appear at 520–540 nm [28]. The increase in absorbance may derive in part from light scattering due to the aggregation.

Secondly, the aggregates do not show any protease-resistant core, unlike fibrils formed by A β [41], Ure2p [42] lysozyme [33] or β 2-microglobulin [43], but are degraded to small fragments in what appears to be a simple one-step mechanism.

Thirdly, the aggregates are narrow and elongated but curly, in contrast to the straight needle-formed structures characteristic of *bona fide* fibrils. The main development from 2 to 96 h is an increase in length of these worm-like structures. Curly aggregates have been seen in other amyloid contexts, for example the salt-induced protofibrils of β 2-microglobulin [44,45]. In the aggregation of a small peptide homologous to the A β peptide, we have previously observed a progression in structure which led from a collection of very curvy threads, akin to a bundle of sewing thread, to a much more straight and rigid collection of needle-formed fibrils [46]. Similarly, Winter and co-workers [47] observed a two-phase fibrillation of insulin under high pressure, where the first phase was characterized by small amorphous aggregates and coagulated elongated structures, whereas the second phase was dominated by networks of straight fibrillar species. In contrast to these proteins, BSA fibrillation appears to be halted at the curly stage, since we did not observe further development in fibrillar structure over longer time scales (data not shown).

Fourthly, although we saw a clear peak at 4.65 Å indicating a typical inter-strand distance seen in classical fibrils [32], there was a multitude of peaks in the region denoting inter-sheet distances (9.5–11 Å). This suggests a mixture of different morphologies rather than a single monolithic structure.

Finally, there is no lag phase in the aggregation process. A lag phase is often attributed to the rate-limiting accumulation of oligomeric structures which nucleate subsequent fibrillation [17]. The absence of such a lag phase can occur if the initial aggregation (which is not highly concentration-dependent in BSA's case) is a down-hill process which does not require a highly organized and unstable nucleus. This appears to be the case for acyl phosphatase, which only accumulates fibrils over a period of several days after an initial lag-free aggregation step [39]. An oligomeric nucleus does not appear to be required for BSA's fibrillation, since the process is not accelerated by seeding of pre-formed aggregates. It is also possible that large multi-domain proteins such as BSA are able to form propagation-competent nucleus-like structures by intramolecular β -sheet formation. This would explain the low concentration dependence of the aggregation process. Such an intermolecular seeding mechanism has been proposed to explain the requirement for sequence diversity between tandem copies of the same protein in β -rich proteins [48]. Clearly BSA's structural rearrangements, which lead to an accumulation of β -sheet structure which can subsequently be propagated by aggregation, occur at the very beginning of the process. This is consistent with our FTIR data which show that a plateau level of β -sheet structure is reached within an hour, similar to Vaiana's observation of the early accumulation of an intermediate with an increased content of β -sheets [24].

Our data thus suggest that BSA is able to form well-ordered β -sheet rich aggregates which nevertheless do not appear to possess the same structural rigidity as classical fibrils. We speculate that the aggregate is held together by a seam of β -sheet structure decorated by relatively disorganized α -helical structure. There is precedence for the co-existence of different types of secondary structure in mature fibrils. Domain-swapped RNase A has been induced to fibrillate by a central extended polyglutamine stretch [49], where the fibrillar core is envisaged to be draped by native RNase A. Similarly, yeast Ure2p fibrils are proposed to be built up of the natively unstructured N-terminal domain, while the structured C-domain retains its native structure [50]. In the case of BSA, however, the presence of these α -helix domains could possibly act as steric blocks against the formation of higher-order structures and thus effectively trap the aggregate at an early stage in the aggregation process as suggested by the EM structures. Such foliage would have several other effects: Firstly, they may prevent optimal β -sheet packing and thus facilitate proteolytic attack. Secondly, they could block the specific formation of the small oligomeric assemblies which are proposed to be cytotoxic by virtue of their membrane-permeabilizing properties [38].

4.2. BSA may form several types of aggregate structures

Generally it is assumed that fibrillation of proteins only occurs at or above T_m , simply because a conformationally flexible state, such as the denatured state, has to be populated to a reasonable degree. This agrees with our observations, since we have to be close to or above T_m (65 °C) for significant fibrillation to occur. Clearly the thermally denatured state is much more efficient at aggregating than the TFE-denatured state, since TFE-denatured BSA does not form aggregates that bind ThT to a level approaching that of the thermally aggregated state (Fig. 7).

Aggregation appears to be able to follow several paths. Fibrillation of BSA can already be detected by the ThT binding assay at 60 °C, whereas the CR assay only detects fibrils at 65 °C and above. In addition, the CR binding assay suggests that a higher activation energy is required for fibrillation than according to the ThT binding assay. Furthermore, a slow increase in ThT binding, but not CR binding, is observed over several days, and is corroborated by EM images showing changes in morphology over this time period. These results indicate that ThT and CR might bind to different fibrillar structures, and consequently that several subtypes of aggregates are formed, possibly in parallel. This would not be without precedent. Mosaic structures of α -synuclein fibrils have been reported [51] and both glucagon [52,53], prions [54] and the A β peptide [55] can form different fibrils depending on solution conditions and sample handling.

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