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Biochemical and Biophysical Research Communications, 2014; 454(2):295-300

Title page

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Hemin as a generic and potent protein misfolding inhibitor

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Abstract

Protein misfolding causes serious biological malfunction, resulting in diseases including Alzheimer's disease, Parkinson's disease and cataract. Molecules which inhibit protein misfolding are a promising avenue to explore as therapeutics for the treatment of these diseases. In the present study, thioflavin T fluorescence and transmission electron microscopy experiments demonstrated that hemin prevents amyloid fibril formation of kappa-casein, amyloid beta peptide, and α-synuclein by blocking β-sheet structure assembly which is essential in fibril aggregation. Further, inhibition of fibril formation by hemin significantly reduces the cytotoxicity caused by fibrillar amyloid beta peptide *in vitro*. Interestingly, hemin degrades partially formed amyloid fibrils and prevents further aggregation to mature fibrils. Light scattering assay results revealed that hemin also prevents protein amorphous aggregation of alcohol dehydrogenase, catalase and γs-crystallin. In summary, hemin is a potent agent which generically stabilises proteins against aggregation, and has potential as a key molecule for the development of therapeutics for protein misfolding diseases.

Highlights

- Hemin prevents Aβ42, α-synuclein, and RCM-κ-casein forming amyloid fibrils
- Hemin inhibits the β-sheet structure formation of Aβ42
- Hemin reduces the cell toxicity caused by fibrillar Aβ42
- Hemin dissociates partially formed Aβ42 fibrils
- Hemin prevents amorphous aggregation by ADH, catalase and γs-crystallin

Keywords

Protein misfolding; Hemin; Amyloid fibrils; Cell toxicity; γs-crystallin

Abbreviations

Aβ42, amyloid-beta peptide 1-42; AD, Alzheimer's disease; ADH, alcohol dehydrogenase; DTT, 1,4-dithiothreitol; RCM-κ-CN, reduced and carboxymethylated kappa-casein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PD, Parkinson's disease; TEM, transmission electron microscopy; ThT, Thioflavin T.

1. Introduction

Most proteins typically fold into unique three-dimensional structures in order to become biologically active [1, 2]. However under stress conditions (elevated temperature, extreme pH, oxidisation etc.), native proteins can misfold via partially structured intermediates to either disordered amorphous aggregates or ordered amyloid fibrils [3]. Amorphous aggregation occurs by a relatively fast and

random process [4-6], whereas amyloid fibril formation occurs in a more ordered manner at a slower rate [7]. Protein misfolding which results in aggregate formation can lead to serious biological consequences. An example of amorphous aggregation is cataract, caused by misfolded crystallin proteins in the eye lens. Age-dependent post-translation modification, such as deamination, oxidation, glycation, and truncation [8-11] of lens crystallin proteins lead to their amorphous aggregation and subsequent precipitation [12] which therefore impair vision. Amyloid fibril formation is associated with more than 20 diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) [13-18]. In AD, the most prevalent age-related neurodegenerative disorder, two proteins aggregate to form amyloid fibrils, namely the amyloid-beta peptide (Aβ) and hyperphosphorylated tau protein [14, 19]. In PD, α-synuclein (αS) is found to be the main protein in amyloid fibrils present in Lewy's body deposits [20-22].

To date, considerable effort has been dedicated to discovering efficacious molecules to combat protein misfolding in order to prevent these diseases or delay their onset. However, there is still no effective, widely used therapeutic to treat protein misfolding diseases. Hemin, the oxidised form of heme, is a crucial component of many physiological processes including electron transport and redox chemistry, and is essential to the function of a number of proteins, such as haemoglobin, cytochrome, catalase and peroxidase [23, 24]. A previous report has shown that hemin prevents Aβ aggregation and reduces cytotoxicity of aggregated Aβ on neuroblastoma cells [25]. However the selectivity and mechanism of hemin as a protein misfolding inhibitor is still unclear. The aims of this research are 1) to evaluate the general efficacy and mechanism of hemin as a protein misfolding inhibitor; 2) to explore the properties of hemin in breaking down preformed, or partially formed fibrils of Aβ42; 3) to investigate the ability of hemin to rescue SH-SY5Y cells from toxicity associated with amyloid fibrils; and 4) to examine the ability of hemin to prevent amorphous aggregation *in vitro*. Therefore this work will provide significant insight into the possibility of developing hemin as an effective therapeutic for preventing or treating protein misfolding diseases.

2. Materials and methods

2.1. Materials

κ-casein (κ-CN) (Sigma, USA) was reduced and carboxymethylated as previously described [26, 27]. The Aβ peptide 1-42 (Aβ42) was purchased from Anaspec (USA), dissolved in 60 μL of 1.0 % NH_4OH and brought to a final concentration of 250 μM using MilliQ water. This stock solution was separated into aliquots and stored at −80 °C until use. α-Synuclein mutant A53T (A53TαS) was expressed and purified as previously described [28]. Hemin, alcohol dehydrogenase (ADH) and catalase were from Sigma. All protein solutions were prepared in phosphate buffer (10 mM, pH 7.4) and passed through a 0.22 μm syringe filter (Pall Corporation, USA) to remove any aggregates prior to experiment. Thioflavin T (ThT), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and 1,4-dithiothreitol (DTT) were obtained from Sigma-Aldrich (Australia). Uranyl acetate was obtained from Agar Scientific (UK). Strong carbon coated 400-mesh nickel grids used for all transmission electron microscopy (TEM) imaging were purchased from ProSciTech (Australia). RPMI1640 powder, foetal bovine serum, horse serum and L-glutamine were purchased from Thermo Electron Corporation (Australia). All other reagents were of analytical grade.

2.2. Thioflavin T assay

ThT fluorescence was measured on a Fluostar Optima plate reader (BMG Labtechnologies, Australia) with a 440/490 nm excitation/emission filter set. The ThT assay was prepared in a 96-well micro-plate in duplicate and incubated in the presence of 10 μM ThT with shaking for A53TαS and without shaking for reduced and carboxymethylated κ-CN (RCM-κ-CN) and Aβ42. 10 μM Synuclein, 25 μM Aβ42 and 25 μM RCM-κ-CN were prepared in phosphate buffer in the absence and presence of 1:1 molar ratio of hemin.

2.3. Transmission electron microscopy

Samples for TEM were prepared by applying 5 μL of protein solution directly from the ThT assays to 400-mesh carbon coated nickel grids, washing three times with 10 μL filtered MilliQ water, then negatively staining using 5 μL 2% (w/v) uranyl acetate. The samples were viewed using a Philips CM100 transmission electron microscope (Philips, The Netherlands).

2.4. Circular dichroism spectroscopy

All far-UV-CD spectra were acquired on a Jasco-715 spectropolarimeter at 25 °C, using a cuvette of 1 mm path length at a scan speed of 10 $nm.min^{-1}$ and a time constant of 0.125 s. Each sample (final concentration 10 μM) was prepared in phosphate buffer (10 mM, pH 7.4). The spectra were recorded in millidegree units over a wavelength range of 190-250 nm then converted and plotted as a function of ellipticity.

2.5. Methyl tetrazolium bromide assay

SH-SY5Y cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium containing 10% v/v horse serum, 5% v/v foetal bovine serum, 10 $U{\cdot}mL^{-1}$ of penicillin and 10 $\mu g{\cdot}mL^{-1}$ of streptomycin and maintained at 37 °C in a humidified incubator with 5 % CO_2. Cells were plated at a density of 2×10^4 cells per well in 96-well plates in 100 μL full-serum fresh medium. After 24 hours, the cells were treated with Aβ42 from the ThT fluorescence assay which was incubated in the absence and presence of hemin, to give a final Aβ42 concentration of 1 μM. Each treatment had six replicates. After a further 48 h of incubation, the treated cells were tested for viability by the MTT assay [29] using a BMG Polarstar microplate reader (BMG Labtechnologies, Germany). The results of the MTT assay were statistically analysed using one-way analysis of variance (ANOVA) followed by a Dunnett's comparison test (GraphPad PRISM V6). Differences were accepted as statistically significant at $p < 0.05$.

2.6. Light scattering assay

Light scattering assays were monitored at 360 nm in a Fluostar Optima plate reader (BMG Labtechnologies, Australia) at 40 °C. Samples for light scattering assays were prepared in a 96 well clear microplate in duplicate, with each well containing 200 μl protein solution either in the absence or presence of a 1:2 molar ratio of hemin.

3. Results and discussion

3.1. Hemin prevents amyloid fibril formation by RCM-κ-CN, Aβ42 and A53TαS

Although amyloid fibril formation is often linked to the onset or progression of a variety of diseases, many non-disease-related proteins can also assemble into amyloid fibrils under appropriate conditions. RCM-κ-CN readily forms amyloid fibrils under physiological conditions *in vitro* [30],

and has proven to be a convenient fibril-forming protein to screen for anti-amyloid compounds due to its robustness and high reproducibility [30]. In the present work, the generic anti-fibril activity of hemin was initially tested on RCM-κ-CN using a ThT assay. ThT is a benzothiazole dye that exhibits enhanced fluorescence upon binding to β-sheet rich structures, and hence is commonly used to monitor amyloid fibril formation [31, 32]. As shown in Figure 1 (A1), the ThT fluorescence profile of RCM-κ-CN incubated in the absence of hemin increased in intensity and reached a plateau after approximately 20 h. When RCM-κ-CN was incubated in the presence of a 1:1 molar ratio of hemin, the ThT fluorescence did not increase with time.

Next, we measured the ability of hemin to prevent the PD and AD related proteins, A53TαS and Aβ42 respectively, forming fibrils. As shown in Figure 1 (B1), the ThT profile of A53TαS incubated in the absence of hemin increased in fluorescence intensity and reached a plateau at 80 h. Similarly, in the absence of hemin, the ThT fluorescence intensity of incubated Aβ42 reached the plateau phase after 6 h (Figure 1 C1). Incubation with hemin prevented ThT fluorescence and hence fibril formation for both disease related proteins.

The increased ThT fluorescence intensity in Figure 1 indicates that amyloid fibrils are formed after incubation for the three proteins studied, which is consistent with TEM images where long mature fibrils are observed (Figure 1:A2, B2, C2). The ability of hemin to prevent fibril formation is also confirmed by TEM images where small aggregates are instead observed, as shown in Figure 1 (A3, B3, and C3).

From these experiments, hemin is shown to prevent a range of peptides/proteins from aggregating to fibrils, and in doing so, converts them into small amorphous aggregate states.

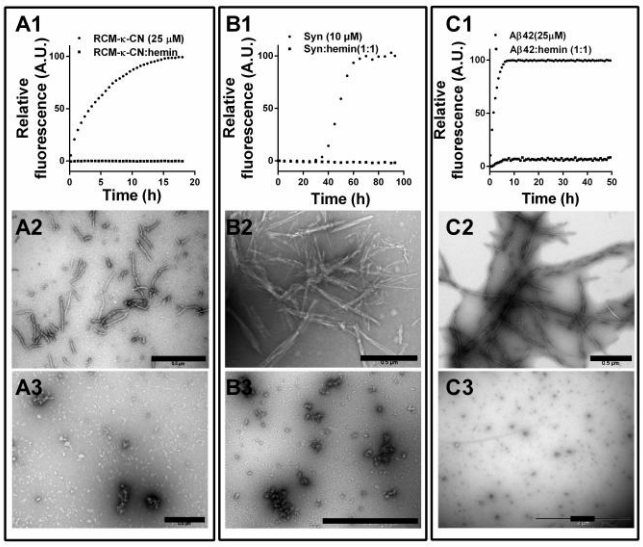

Figure 1: Inhibitory effects of hemin on the amyloid fibrillar aggregation of RCM-κ-CN, A53TαS and Aβ42. (A1) Time-dependent ThT fluorescence of 25 μM RCM-κ-CN incubated in 100 mM phosphate buffer (pH 7.4) at 37 °C without shaking in the absence and presence of hemin, and TEM images of RCM-κ-CN fibrils formed after incubation in the (A2) absence and (A3) presence of hemin; (B1) Time-dependent ThT fluorescence of 10 μM A53TαS incubated in 100 mM phosphate buffer (pH 7.4) at 37 °C with shaking in the absence and presence of hemin, and TEM images of A53TαS fibrils formed after 100 h incubation in the (B2)

absence and (B3) presence of hemin; (C1) Time-dependent ThT fluorescence of 25 μM Aβ42 incubated in 100 mM phosphate buffer (pH 7.4) at 37 °C without shaking in the absence and presence of hemin, and TEM images of Aβ42 fibrils formed after incubation for 48h in the (C2) absence and (C3) presence of hemin. Scale bar = 500 nm.

Potency of hemin to prevent RCM-κ-CN fibril formation was compared with that of EGCG, which is a widely accepted inhibitor of fibril formation by the ThT assay. The IC50 of hemin is 1.4 ± 0.18 μM compared to 12.8 ± 1.5 μM of EGCG, which indicates that hemin is a potent inhibitor to amyloid fibril formation.

3.2. Hemin prevents Aβ42 β-sheet structure formation

Cross-β-sheet structure conversion is closely linked with the process of amyloid fibril formation [33, 34]. The cross-β structures have either parallel or anti-parallel orientations of stacked β-sheet monomers aligned perpendicular to the fibril axis [35]. The secondary structure of Aβ42 before and after fibril formation in the presence and absence of hemin was analysed using far-UV CD spectroscopy to probe for β-sheet secondary structure. As shown in Figure 2A, before incubation, Aβ42 gives a strong negative ellipticity reading at 195 nm, indicating the presence of a largely unfolded, random structure. After 50 h of incubation at 37 °C, the Aβ42 solution produces a far UV-CD spectrum with a broad absorption minimum at 217 nm, arising from a stabilization of β-sheet structure. Due to the aggregation of Aβ42, less soluble peptide was left in the solution therefore leading to the reduction in intensity and decreased the signal to noise ratio in the observed profile (red line). The spectrum of incubated Aβ42 in the presence of hemin exhibits features with a minimum ellipticity at approximately 195 nm, the same as before incubation (Figure 2A), implying that hemin maintains the random-coil conformation of Aβ42 by blocking the formation of β-sheet rich intermediates.

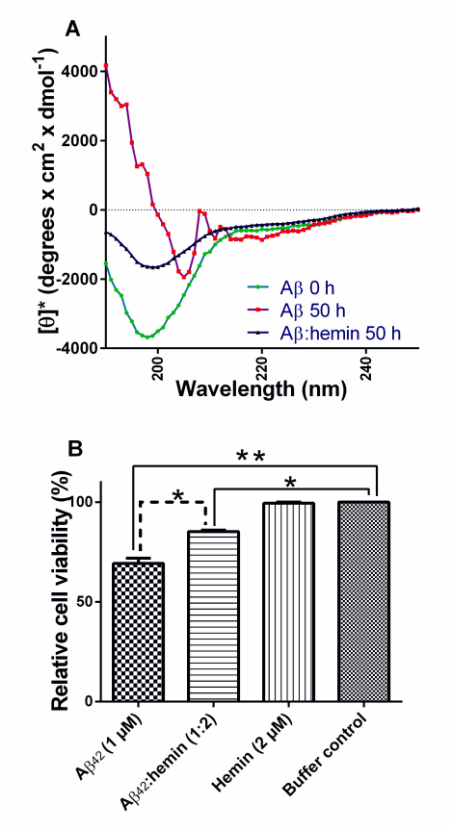

Figure 2: A: Secondary structure determination of Aβ42 by far-UV CD. Aβ42 (25 μM) in 10 mM phosphate buffer (pH 7.4) was incubated at 37 °C in either the absence of hemin (t=0 h green line, t=50 h red line) or presence of hemin (blue line) at a 1:2 molar ratio for 50 h. **B**: **Cytotoxity induced by incubated Aβ42 as determined by the MTT assay**. 25 μM Aβ42 was incubated in 100 mM phosphate buffer (pH 7.4) in the presence and absence of hemin for 50 h before being used to treat cells after dilution to the indicated concentration. Data are an average of three experiments and error bars indicate SEM expressed as percentages relative to control cells treated with buffer. * and ** refer to a significant difference ($p < 0.05$) and highly significant difference ($p < 0.01$) respectively.

Further experiments were conducted to investigate the interaction of hemin with Aβ42 using soft ionisation electrospray mass spectrometry. Unfortunately, no detectable and stable complexes were observed when Aβ42 was incubated with hemin (Supplementary figure 1), implying that any interaction between hemin and Aβ42 is weak and transient in nature. This is consistent with the interaction of Aβ42 and other amyloid fibril forming peptides and proteins with their inhibitors, e.g. α-synuclein with the molecular chaperone αβ-crystallin and α-synuclein with gallic acid [36][27].

3.3. Hemin reduces the cytotoxicity of aggregated Aβ42

In our present work, the toxicity of incubated Aβ42 was evaluated on SH-SY5Y cells, a cell line often used as an model of neuronal function and differentiation [37]. In particular, the ability of hemin to prevent the cytotoxicity associated with incubated Aβ42 was examined using an MTT assay. 25 μM Aβ42 was pre-incubated overnight at 37 °C in the absence and presence of 50 μM hemin before exposure to SH-SY5Y cells. After serial dilution, a final concentration of 1 μM of incubated Aβ42 was added to SH-SY5Y cells either in the presence or absence of 2 μM hemin. The results showed that the viability of SH-SY5Y cells exposed to incubated Aβ42 in the absence of hemin reduced to 69.3 ± 2.7 %; a significant reduction compared to buffer control ($p < 0.01$). The viability of cells treated with incubated Aβ42 in the presence of hemin increased to 85.2 ± 0.8 % (Figure 2B), a significant improvement compared to fibrillar Aβ42 ($p < 0.05$). These results demonstrate that the inhibition of fibril formation of Aβ42 as a result of hemin blocking β-sheet structure transformation leads to a reduction of cell toxicity. It has previously been shown that hemin is toxic to PC12 cells and SH-SY5Y with a LD_{50} of 25 μM [38]. However, in our present study, 2 μM of hemin did not show any toxic effects, as indicated in Figure 2B, implying that hemin is safe at the tested concentration.

3.4. Hemin dissociates partially formed Aβ42 fibrils

We investigated the ability of hemin to break down preformed amyloid fibrils. After 25 μM Aβ42 peptide was incubated for 20 h, hemin was added to the incubation solution at a molar ratio of 1:8 and further incubated for 30 h. As shown in Figure 3A, ThT fluorescence intensity dropped immediately after addition of hemin and remained low with further incubation. TEM images corresponding to Aβ42 incubated for 20 h shows a mixture of short and long filaments indicating that the fibrillization process is not fully complete at this time point (Figure 3B). After a further 30 h incubation in the absence of hemin, only long filaments are observed (Figure 3C) suggesting that all fibrils are matured. After a further 30 h incubation in the presence of hemin, the short, partially formed fibrils which were present in Figure 3B disappeared completely, indicating that hemin can break down the partially formed amyloid fibrils into soluble protein, or convert them to amorphous aggregates which can be viewed in Figure 3D.

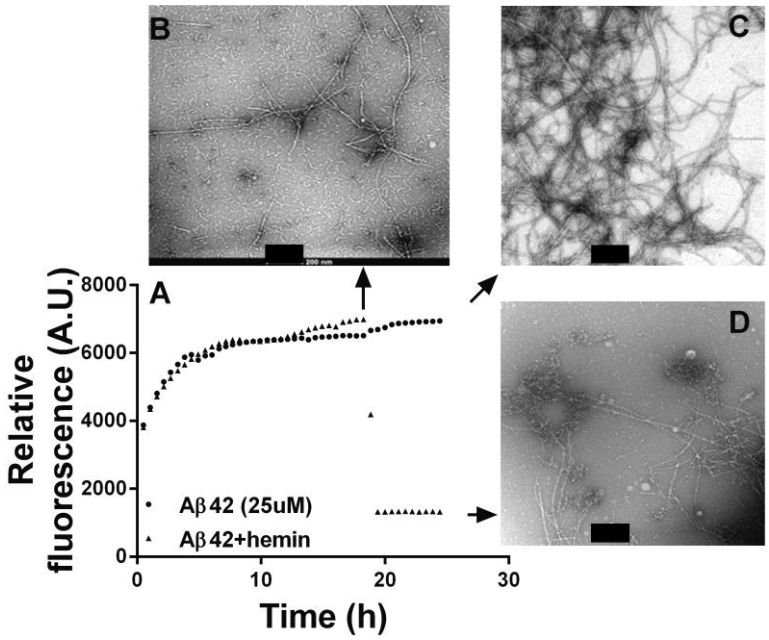

Figure 3: Dissociation effects of hemin on preformed Aβ42 fibrils. (A) Time-dependent ThT fluorescence of 25 μM Aβ42 with hemin added after incubation for 20 h in 100 mM phosphate buffer (pH 7.4) at 37 °C; (B) TEM image of 25 μM Aβ42 after incubation for 20 h; (C) TEM image of 25 μM Aβ42 after incubation for 50 h; (D) TEM image of 25 μM Aβ42 after incubation for 50 h with hemin added at 20 h incubation at a 1:8 molar ratio. Scale bar = 200 nm.

Reversible fibril formation has been reported for several fibrillar proteins and peptides [39, 40]. An *in vivo* study revealed that fibril formation of Aβ42 is initiated by nucleation, followed by reversible deposition, then by irreversible fibrillization [39]. In the present study, it is clear that hemin can interact with and degrade the partially formed fibrils corresponding to the reversible aggregates. When the fibrils reach an irreversible state, hemin cannot dissociate them (Figure 3D). This result suggests the possibility for use of hemin as a therapeutic agent to clear partially formed plaques before amyloid fibrils are fully formed.

3.5. Hemin inhibits amorphous aggregation

Different from amyloid deposits, which can be measured using ThT assays and can be distinguished using TEM technology, amorphous aggregate formation is normally monitored via turbidity measurements. In the present study, to evaluate the ability of hemin to prevent amorphous aggregation, we chose catalase, ADH and γs-crystallin as target proteins whose aggregation can be induced thermally. As shown in Figure 4 A and 4B, the aggregation of catalase and ADH reached a maximum after incubation at 40 °C, yet in the presence of hemin, the turbidity associated with their precipitation was totally suppressed.

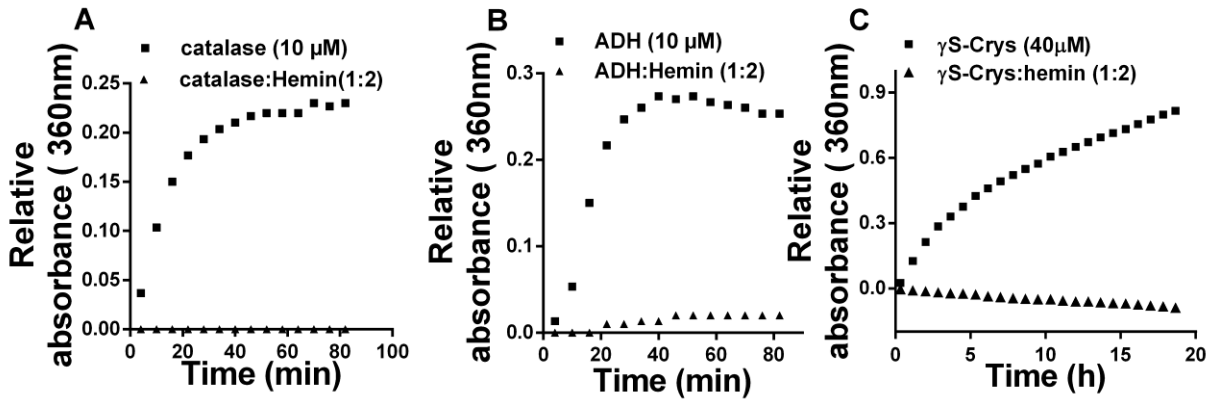

Figure 4: Effects of hemin on amorphous aggregation as measured by light scattering. Solution turbidity following incubation was monitored at 360 nm. 10 μM catalase (A) or ADH (B) or 40 μM γs-Crystallin (C) in 100 mM phosphate buffer, pH 7.4 was incubated at 40 °C in the absence (■) or presence of hemin (▲) in duplicate.

Hemin is also effective in inhibiting amorphous aggregation of γs-crystallin. γcrystallin is one of lens structural proteins with seven members, γA to γF and γS. Like other crystallin proteins, γs-crystallin must remain stable and soluble for the transparency of the eye lens. While aggregation of this protein leads to cataract clinically [41]. *In vitro* aggregation of γs-crystallin was thermally induced in the present study. And aggregation of γs-crystallin was prevented in the presence of hemin (Figure 4 C), which indicates that hemin can be investigated further for preventing or treating cataract.

4. Conclusions

In summary, we have demonstrated that hemin can prevent both amorphous aggregation and amyloid fibril formation for a variety of proteins, suggesting that hemin is a generic protein misfolding inhibitor. The toxicity of incubated Aβ42 to SH-SY5Y cells can be attenuated by inhibiting fibril formation utilizing hemin, which highlights the importance of hemin in inhibiting the cell toxicity associated with fibril formation in protein misfolding diseases. Moreover, hemin breaks down partially formed amyloid fibrils of Aβ42, which indicates that hemin can be used to prevent the progress of misfolding disease. Consequently, although the anti-aggregation and fibril degrading mechanisms of hemin are not known at a molecular level, hemin could be a key molecule for the development of therapeutics for protein misfolding diseases.

Acknowledgements

The authors thank the Australian Research Council for financial support of this investigation. Assistance provided by Ms Lyn Waterhouse for transmission electron microscopic analysis is gratefully acknowledged. γs-crystallin was a gift from Dr Damien Hall (The Australian National University).

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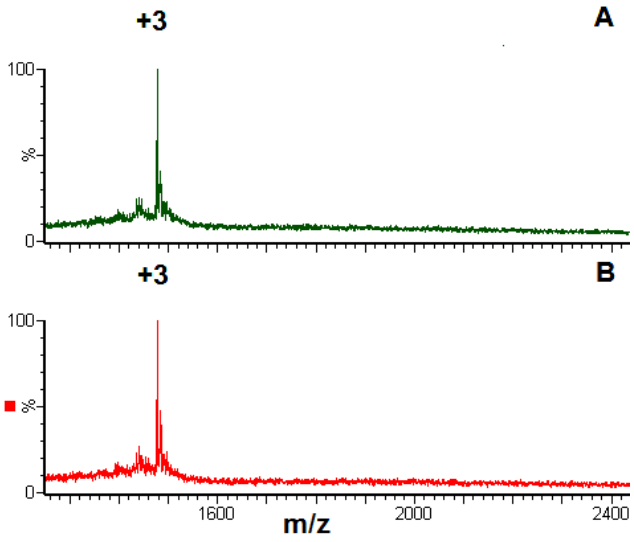

Supplementary figure 1: Q-TOF mass spectra of 25 µM Aβ42 in 50 mM ammonium acetate buffer in the absence (A) and presence of 50 µM of hemin (B).