



Review Article

Studying the effects of chaperones on amyloid fibril formation

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ARTICLE INFO

Article history:

Available online 7 December 2010

Keywords:

Amyloid

Prion

Chaperone

Thioflavin T

Biosensor assay

Filter retardation assay

A B S T R A C T

The results of cell and animal model studies demonstrate that molecular chaperones play an important role in controlling the processes of protein misfolding and amyloid formation *in vivo*. In addition, chaperones are involved in the appearance, propagation and clearance of prion phenotypes in yeast. The effect of chaperones on amyloid formation has been studied in great detail in recent years in order to elucidate the underlying mechanisms. An important approach is the direct study of effects of chaperones on amyloid fibril formation *in vitro*. This review introduces the methods and techniques that are commonly used to control and monitor the time course of fibril formation, and to detect interactions between chaperones and fibril-forming proteins. The techniques we address include thioflavin T binding fluorescence and filter retardation assays, size-exclusion chromatography, dynamic light scattering, and biosensor assays. Our aim in this review is to provide guidance on how to embark on study of the effect of chaperones on amyloid fibril formation, and how to avoid common problems that may be encountered, using examples and experience from the authors' lab and from the wider literature.

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1. Introduction

Amyloid fibril formation is implicated in over 20 different human diseases, including Alzheimer's disease, Creutzfeldt–Jakob disease and type II diabetes, and is also associated with prion phenomena in fungi [1,2]. Amyloid is defined in terms of empirical observations from X-ray fibre diffraction, morphological determination (such as by electron microscopy, EM) and specific chemical staining (such as with Congo red or thioflavin T, ThT). There are three classical criteria that together define amyloid fibrils: cross- β structure, fibrillar morphology and green birefringence upon Congo red staining [3–5]. Amyloid fibrils are often also quite stable to protease K digestion and detergents. With respect to the nucleation mechanism of fibril formation, a typical time course for amyloid fibril formation is composed of three stages: a lag phase of slow growth corresponding to seed formation, a logarithmic phase of rapid growth corresponding to fibril elongation, and a plateau phase of stationary growth, during which there may be continuing morphological changes, such as fibril assembly from proto-fibrils to mature fibrils (Fig. 1). The time course of fibril formation can

be followed using specific chemical staining combined with morphological observations [6].

As part of the protein quality control system, chaperones help proteins to fold correctly and prevent misfolding [7]. Chaperones can also take part in disassembly of protein aggregates *in vivo* and *in vitro* [8,9]. Chaperones are thought to act as protective agents against amyloid diseases and as regulators of prion phenomena. Overexpression of chaperones of the Hsp70 and Hsp40 families has been reported to rescue cells from the toxicity caused by amyloid deposits [10,11]. In fungi, overexpression, deletion or mutation of some chaperones has been found to cure prion phenotypes [2,12]. Chaperone-dependent effects on amyloid fibril formation can be studied by *in vivo* or *in vitro* experimentation. *In vivo* experiments give information on how chaperones regulate amyloid fibril formation in cooperation with other cell factors, while *in vitro* experiments provide direct evidence about the interaction between chaperones and fibril-forming proteins. In this review we focus on *in vitro* methods.

When studying chaperone-dependent effects on amyloid fibril formation *in vitro*, two questions may be posed. (1) What is the direct effect of chaperones on fibril formation? (2) What are the mechanisms of the effects of chaperones on amyloid fibril formation? To address the first question, monitoring the effect of chaperones on the time course of fibril formation using specific chemical dyes (such as ThT) has often been combined with morphological observations for evaluating the extent of the effect of chaperones (such as by EM or atomic force microscopy, AFM). Measuring the

Abbreviations: AFM, atomic force microscopy; DLS, dynamic light scattering; EM, electron microscopy; QCM, quartz crystal microbalance; SEC, size-exclusion chromatography; SPR, surface plasmon resonance; ThT, thioflavin T.

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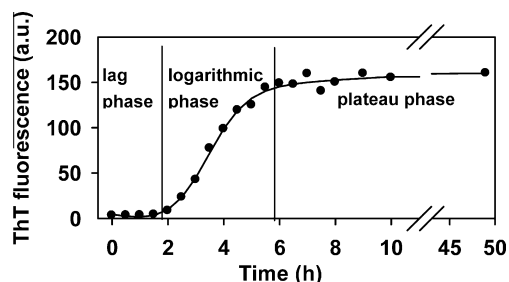


Fig. 1. The phases of fibril growth observed by ThT binding fluorescence. The figure shows the time course of Ure2 fibril formation [6]. The figure was adapted with permission from the publisher.

interaction between chaperones and fibril-forming proteins can provide evidence to address mechanisms underlying the chaperone effects. More detailed information on these mechanisms can be obtained using mutants of either the chaperones or the fibril-forming proteins.

2. Monitoring the effect of chaperones on the time course of fibril formation

2.1. Choosing experimental conditions for monitoring fibril formation with chaperones

Experimental conditions required for fibril formation may vary depending on the fibril-forming protein being analysed, and a number of different experimental conditions may be suitable for fibril formation of a particular protein. Optimising experimental conditions is the first step in studying mechanisms of fibril formation and the effects of chaperones. The choice of experimental conditions is often limited by the need to maintain chaperone activity and also the need to control the total duration of the experiment. On the one hand, the course of fibril formation must be slow enough to allow the different phases of fibril growth to be distinguished (Fig. 1), in order to clarify at what stage(s) during the time course the chaperones exert their effect. On the other hand, the time course must be completed within a reasonable timescale to allow convenient monitoring and completion of the experiment. Experimental timescales and fibril formation rates can be controlled by fine adjustment of various experimental conditions, as outlined below. It should be noted, however, that variations in fibril growth conditions can lead to differences in fibril structure for a given protein [13], which in turn could affect interaction with chaperones. Therefore, in general, a number of different experimental conditions should be sampled and compared. Experimental conditions that are readily varied include the following:

2.1.1. Choice of buffer conditions

Variables such as pH, ionic strength and whether to include a reducing agent must all be considered. A good buffer is one that maximises protein stability and solubility and also prevents the formation of amorphous aggregates, since formation of fibrils often competes with formation of amorphous aggregates [14]. Even at a given pH, different buffers may have different effects on protein stability and fibril formation. For example, the yeast prion protein Ure2 is substantially less stable in phosphate buffer than in Tris buffer at the same pH [14]. It is worth investigating the effects of different buffer conditions on fibril formation for a given protein. Buffers suitable for fibril formation with chaperones are generally around neutral pH and containing 100–200 mM salt, in order to mimic the physiological environment. Buffer conditions should ensure the normal function of chaperones. Some chaperones

require special salts for their chaperone activity. For example, Hsp70 requires KCl and MgCl₂ for its ATPase activity [15]. Low concentrations of reducing agents (e.g. 1 mM DTT) are generally added when cysteines are present in the chaperone. For Hsp70 from most species, addition of 1 mM DTT (or 5–10 mM β-mecaptethanol) is essential for maintaining its normal function. Higher concentrations of reducing agent are used when the oxidation or reduction of cysteines in the fibril-forming protein determine the speed of fibril formation. For example, fibril aggregation of insulin is dependent on the reduction of disulfide bonds [16], and fibril formation of the Ure2 mutant R17C is accelerated by oxidizing agents and decelerated by reducing agents [17]. ATP and ATP regeneration systems should be included for chaperones with ATPase activity, although in some cases, addition of ADP, AMP or a slowly-hydrolyzable analogue such as ATP-γS can be used to capture different functional states of chaperones [18–20].

2.1.2. Choice of temperature

The temperature may be chosen to mimic physiological conditions (i.e. 37 °C for mammalian or *E. coli* systems, or up to 30 °C for yeast). In general, lower temperatures will tend to slow down fibril formation, therefore temperatures are often varied between 4 and 37 °C, in order to optimize the rate of fibril formation, depending on the requirements of the experiment.

2.1.3. Choice of incubation conditions: with or without agitation

When the tendency to form fibrils in a given fibril system is very strong, incubation alone is sufficient to promote fibril formation. Shaking or stirring can be utilized if the rate of fibril formation on standing is very slow, or in order to give more reproducible rates of fibril formation. Shaking is more convenient and easily controlled than stirring, and the rate of fibril formation is readily adjusted by altering shaking speeds. Shaking in the presence of one or more glass or Teflon beads tends to further accelerate the rate of fibril formation, presumably by promoting fragmentation of fibrils to produce more termini from which fibril growth can occur.

2.1.4. Monomeric concentration of the fibril-forming protein

In general, the rate of fibril formation is positively correlated with the concentration of monomers of the fibril-forming protein in the fibril system. If the protein concentration is too low, fibrils may not form at all, or this may limit detection of fibril formation. A series of concentrations can be evaluated to choose the most suitable concentration for observing the effect of chaperones on fibril formation.

2.1.5. Addition of pre-formed fibril seeds

The addition of pre-formed fibril seeds can accelerate fibril formation and shorten the lag time. Seeds can also be used to induce the monomeric proteins to form fibrils of the same structure as the seeds [13,21,22]. Fibril seeds are generally produced by sonication of the fibrils (see Section 3.3.1 below).

2.2. ThT binding fluorescence measurements

There are at least 10 different chemical dyes that are used as molecular probes for amyloid fibril detection, of which ThT is the

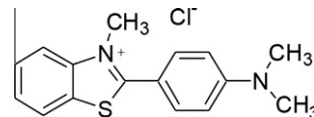


Fig. 2. Chemical structure of Thioflavin T.

most commonly used. ThT is a cationic benzothiazole dye (Fig. 2) that shows enhanced fluorescence upon binding to amyloid both *in vivo* [23] and *in vitro* [24]. Emission is generally monitored at 482 nm after excitation at 450 nm [24]. Binding of ThT to DNA and RNA also demonstrates enhanced fluorescence [23], and in general, ThT will tend to bind to a number of types of ordered aggregates, including elements of the cytoskeleton. Some proteins can show non-specific binding to ThT under certain experimental conditions [25]. Therefore, the inclusion of appropriate controls is important.

In recent years significant attention has been paid to trying to understand the molecular mechanism of ThT binding to amyloid fibrils. It is known that ThT can bind to the β -strands of amyloid fibrils [26]. A high-resolution structure of the complex between amyloid and ThT has not yet been obtained, but binding of individual molecules of Congo red and ThT to A β fibrils has been observed by scanning tunneling microscopy [27]. However, a clear picture of the ThT binding mechanism is not available and the K_d and fluorescence yield on binding of ThT to amyloid fibrils formed from different proteins is found to vary [28,29]. In addition, the concentration of ThT, buffer conditions of the ThT solution, and the fibril concentration all contribute to the final ThT fluorescence yield. Before monitoring the time course of chaperone-dependent fibril formation by ThT binding fluorescence, experimental conditions for detecting the target fibrils by ThT binding fluorescence should be optimized as follows:

2.2.1. Choice of ThT concentration

It is still unclear whether the molecular form of ThT which binds to amyloid fibrils is monomeric, dimeric or micellar. If ThT binds to fibrils as a micelle, the concentration of ThT should be over 4 μ M, which is the critical concentration for ThT to form micelles [30]. If the ThT concentration is much higher than the monomer concentration of the fibril-forming protein, this will help ensure that most of the fibrils will be bound by ThT. The ThT binding fluorescence is measured as the ratio of ThT fluorescence upon binding to fibrils to that of the fibril-free blank. Some fibrils (such as Ure2 fibrils, insulin fibrils and A β fibrils) only require relatively low concentrations of ThT (lower than 10 μ M) to yield satisfactory ThT binding fluorescence. However, other fibrils, like those formed from poly-Q proteins, require a relatively high ThT concentration (over 20 μ M) to obtain accurate measurements.

2.2.2. Choice of buffer conditions

Neutral pH is commonly used. However, some fibrils give optimal ThT binding fluorescence at a pH other than neutral [25]. The choice of pH used will be a balance of optimizing the fluorescence yield with ensuring that the proteins studied are in an active and stable conformation. Salt concentration has also been found to affect the K_d of ThT binding to fibrils [31].

2.2.3. Choice of monomeric concentration of the fibril-forming protein

The initial protein concentration will affect both the rate of fibril formation and the amount of fibrils formed. In general, the fluorescence intensity increases approximately linearly to the amount of fibrils in the ThT binding fluorescence detection system (although a strict linear relationship cannot be assumed and must be tested for a given protein, if this relationship is important for interpretation of the data). Therefore the choice of initial protein concentration can be adjusted to control the duration of the experiment and to optimize the ThT fluorescence signal.

2.2.4. Protocol for ThT fluorescence assay measurements

ThT fluorescence measurements upon binding to amyloid fibrils can be carried out according to the following procedure:

- (1) Prepare a ThT stock solution by adding 1–3 mg ThT dry powder to 1 ml water. The solution should be filtered through a 0.22 μ m filter membrane. The stock can be kept at 4 °C for several months if protected from light (e.g. by wrapping with foil). Re-filtering is essential before use since some ThT may precipitate after a long period of storage. The concentration of the ThT stock can be calculated by measuring the absorbance at 416 nm by diluting the stock solution (e.g. 100-fold) into ethanol and using an extinction coefficient of 26,620 M⁻¹ cm⁻¹ [32].
- (2) Dilute the stock solution with the optimized buffer to obtain the desired ThT concentration and generate a working solution.
- (3) Add 10–20 μ L of the fibril sample to achieve a working system with a total volume of 600 μ L (in the control, the same volume of buffer should be added in place of the fibril solution).
- (4) After mixing the working solution and allowing it to stand for 1 min, transfer the working system to a quartz fluorescence cuvette. Measure the fluorescence intensity by excitation at 450 nm and emission at 482–490 nm, averaging over 10–60 s. The slit width can be adjusted from 5 to 10 nm according to the fluorescence intensity needed. The fluorescence of the control (no fibrils) should be subtracted from the readings for each sample.

2.2.5. Monitoring the time course of fibril formation

Fibril samples can be incubated in individual microcentrifuge tubes, and aliquots removed for analysis, as described above. Measurements can also be performed in multi-well plates and/or *in situ*. *In situ* experiments require the choice of a buffer that can be used for both fibril formation and ThT assays; but in this case there is the concern that the presence of ThT during fibril growth will influence the growth kinetics. In general, the final result should be the mean of at least three parallel replicates because of the error involved in fluorescence measurements, whichever kind of detection mode is applied.

The time course of fibril formation can be monitored by detecting ThT binding fluorescence at regular time intervals. This is straightforward in *in situ* experiments, and the spectrophotometer can read the fluorescence value whenever required. If fibril formation and ThT fluorescence assays are separated in time, sample aliquots can be taken from the fibril formation system at regular time intervals. Time intervals should be short during the lag and logarithmic phases (to provide enough data points during these two phases to increase the reliability of the time course curve), but they can be lengthened during the plateau phase.

2.2.6. Monitoring ThT fluorescence in the presence of chaperones

The effect of chaperones on fibril formation can be analyzed by comparing time courses with or without chaperones. If a chaperone influences fibril formation then the degree of the effect observed will generally depend on the chaperone concentration. The performance of the time course at a series of different chaperone concentrations (Fig. 3) can give information about the stoichiometry or mode of binding between the chaperone and the fibril-forming protein [33,34]. An increase in the lag time in the presence of the chaperone suggests interaction with the native state of the fibril-forming protein or with intermediates formed early in the time course of fibril formation. In some cases, the magnitude of the ThT fluorescence may give an indication of the extent of fibril formation; but this requires caution, as the absolute fluorescence value can be influenced by a number of factors. Preliminary investigations of the mechanism by which chaperones affect fibril formation can be conducted by adjusting the stage in the time course at which chaperones are added to the fibril formation

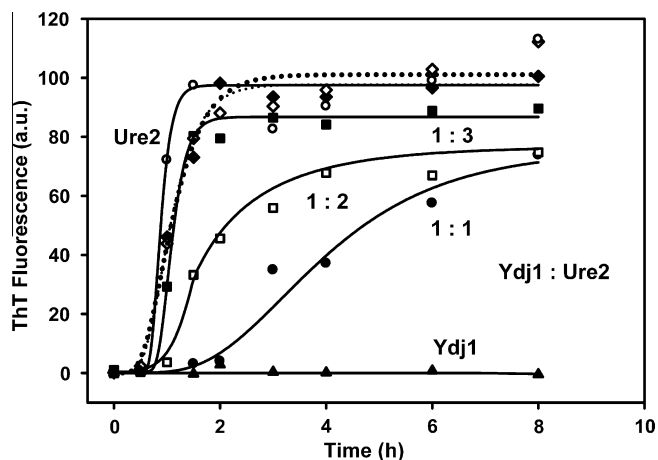


Fig. 3. Ydj1 concentration-dependent inhibition of Ure2 fibril formation, monitored by ThT binding. Conditions were 50 mM Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl with shaking at 37 °C. Fibril formation of 30 μM Ure2 in the absence (○) and presence of Ydj1 (3 μM, ◇; 7.5 μM, ◆; 10 μM, ■; 15 μM, □; 30 μM, ●). As a control, 30 μM Ydj1 alone (▲) was incubated under the same conditions. The results indicate that Ydj1 inhibits fibril formation in a stoichiometric (rather than a catalytic) manner, and imply that the mechanism of inhibition may involve a stable interaction between the proteins [33]. The figure was reproduced with permission from the publisher.

system. The effect of chaperones added at different time points (Fig. 4) can be compared to distinguish which intermediates formed during the course of fibril formation are able to interact with chaperones [33–35]. It should be noted that the volume of chaperone solution added should be as small as possible if it is not added at the beginning of the experiment, as changes in volume may affect fibril formation (in the control, the same volume of buffer should be added).

Fibril formation time courses obtained by monitoring ThT fluorescence can be confirmed by conducting AFM or EM measure-

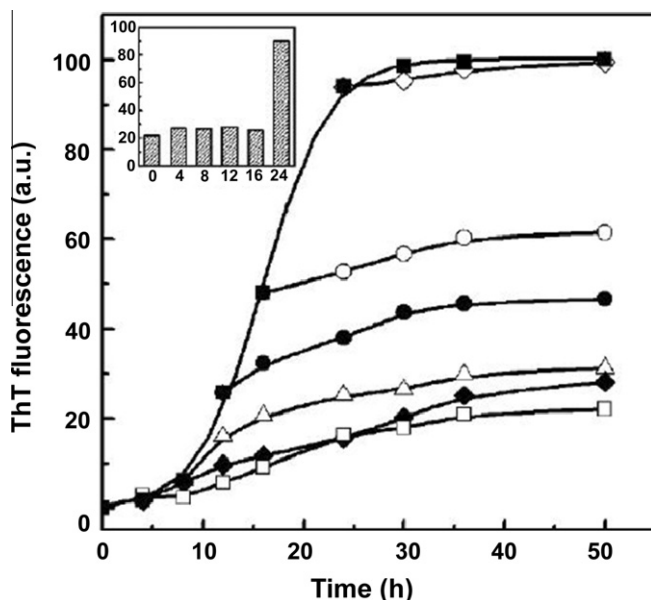


Fig. 4. Inhibition of alpha-synuclein fibril formation by delayed addition of Hsp70. Fibril formation of 70 μM alpha-synuclein in the absence (■) and presence of 35 μM Hsp70 added at 0 (□), 4 (◆), 8 (△), 12 (●), 16 (○) and 24 (◇) h was measured by ThT fluorescence. Insets show the percentage of the increased ThT fluorescence at 50 h after addition of Hsp70 to that with no Hsp70 added. The results indicate that Hsp70 can interact with a variety of intermediates of alpha-synuclein [35]. The figure was reproduced with permission from the publisher.

ments [33,34]. This is important, as the ThT fluorescence assay can give false positives, such as non-specific ThT fluorescence arising from the binding of ThT to native proteins or amorphous aggregates. ThT fluorescence can also give false negatives, for example when the ThT binding of some fibrils under certain conditions is sub-optimal and therefore not easily detected [6]. In order to interpret the results of ThT binding assays in terms of the effect of the presence of chaperones, it is essential to determine: (1) the ThT binding fluorescence of native fibril-forming proteins and chaperones. (2) The time course when chaperones alone are incubated under the same conditions as for fibril formation. The time course of fibril formation monitored by ThT binding fluorescence should always be checked for consistency with that obtained by EM or AFM.

2.3. Filter retardation assays

Fibrils can be separated according to their detergent resistance and size, and then detected by immunological methods [36]. In the filter retardation assay, SDS-treated fibrils are filtered through a 0.22 μm cellulose acetate membrane. The amount of fibril on the membrane is then measured using an appropriate fibril antibody as the primary antibody. The time course of fibril formation can be monitored by taking sample aliquots from the fibril formation system at regular time intervals. Currently the best membranes to use for filter retardation assays are cellulose acetate membranes because of their low tendency to adsorb proteins. The experimental conditions for the dot-blot step can be optimized according to the basic protocols for Western blotting. This method has not been used as widely as ThT binding fluorescence because the manipulations involved are not as straightforward, particularly for an inexperienced user. The method is often used for detecting poly-Q fibrils [37,38] (Fig. 5) and fibrils formed *in vivo* [39,40], since the ThT assay is not very sensitive for poly-Q fibrils and is not suitable for fibrils mixed with cell components. In terms of the limitations of this technique, it should be noted that the SDS-resistance of some fibrils (such as Ure2 fibrils) is low and the membrane may not capture small aggregates formed during the early stages of fibril formation.

Filter retardation assays can be carried out according to the following procedure [41]:

- (1) Heat 100–500 ng of amyloid fibrils in 2% SDS and 50 mM DTT (in a volume of 50–100 μL) at 98 °C for 3 min.
- (2) Pre-equilibrate a cellulose acetate membrane with 0.1% SDS.
- (3) Lay the pre-equilibrated membrane on a dot-blot filtration unit (such as a Bio-Dot apparatus, Bio-rad).
- (4) Filter the SDS-treated fibrils through the cellulose acetate membrane.
- (5) Wash twice with 200 μL 0.1% SDS and remove the membrane from the dot-blot filtration unit.
- (6) Block the membrane with blocking solution for 1 h at room temperature.
- (7) Wash three times in TBS (50 mM Tris, pH 7.5 containing 150 mM NaCl) or phosphate buffered saline (PBS) containing 0.05% Tween 20 for 10 min.
- (8) Incubate the membrane with the fibril antibody diluted in TBS (or PBS) containing 0.05% Tween 20 for 2 h at room temperature.
- (9) Wash three times in TBS (or PBS) containing 0.05% Tween 20 for 10 min.
- (10) Incubate the membrane with a secondary peroxidase-conjugated antibody for 1 h at room temperature.
- (11) Wash the membrane twice in TBS (or PBS) containing 0.05% Tween 20 for 5 min, and then twice in TBS (or PBS) for 10 min.

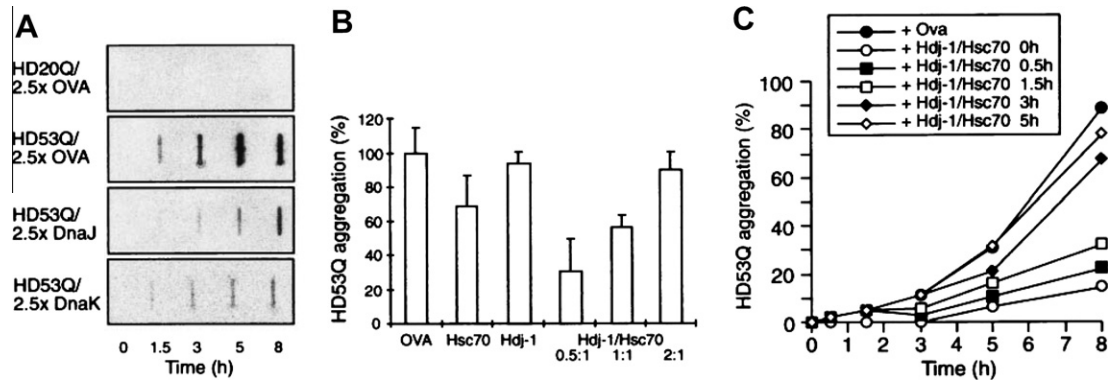


Fig. 5. Modulation of aggregation of Huntingtin exon 1 by Hsp70 and Hsp40 *in vitro*. The figure was reproduced from [38] with permission, copyright (2000) National Academy of Sciences, USA. (A) Time-dependent formation of SDS-insoluble aggregates of HD20Q and HD53Q (3 μ M) in the presence and absence of ovalbumin (OVA) and DnaJ (Hsp40) or DnaK (Hsp70) of *E. coli* (7.5 μ M each) in the absence of ATP as detected in filter retardation assays. Chaperones were added when aggregation was initiated by proteolytic cleavage of GST-HD fusion proteins. OVA served as a non-chaperone control protein. (B) Cooperation of mammalian Hdj-1 (Hsp40) and Hsc70 proteins in retarding SDS-insoluble HD53Q aggregation in the presence of ATP (2 mM). The ratio of Hdj-1 to Hsc70 was varied with Hsc70 and HD53Q at 3 μ M. Amounts of aggregates after 8 h of incubation were quantified by Phosphorimager densitometry. Means and standard deviations of at least three independent experiments with similar results are shown. (C) Time course of formation of SDS-insoluble HD53Q aggregates with addition of Hsc70/Hdj-1/ATP (6 μ M/3 μ M/2 mM) occurring at different times after initiating aggregation by proteolytic cleavage of GST-HD53Q (3 μ M).

- (12) Incubate the membrane with ECL (enhanced chemiluminescence) reagent for 3 min in the dark at room temperature. Expose the membrane to light-sensitive film or photograph the membrane directly with a fluorescence imager.
- (13) Quantify the amount of captured aggregates by scanning the dot-blot image and analysing with appropriate software.

3. Detection of interactions between chaperones and fibril-forming proteins

3.1. Size-exclusion chromatography

Size-exclusion chromatography (SEC) is a method that can allow direct observation of stable interactions between chaperones and the native state or small oligomers of amyloid-forming proteins. If the chaperone forms a stable complex with the amyloid-forming protein, an alteration in the retention time of the relevant species on the column will be observed. The column is first equilibrated with buffer before injecting the chaperone and the amyloid-forming protein separately, to determine the peak position (or retention time or volume) for each of the individual species. The protein mixture is then injected, and the peaks can be compared. Alteration in the positions of the peaks indicates an interaction between the proteins. While SEC is relatively easy to perform, it can only detect complexes between chaperones and amyloid-forming proteins where there is a relatively high affinity between the proteins; if the interaction is weak or transient, changes to positions of the peaks may not be detected. On the other hand, if no change in the peak positions is detected, it cannot necessarily be concluded that there are no interactions present, and other approaches should be taken to further investigate the interaction.

The preincubation time and temperature of the mixture before injection onto the column is an important variable that may affect whether or not an interaction is observed. The relative molecular weights of the chaperone and the amyloid-forming protein is another factor that must be considered for this technique. If they are very similar in size, the peaks of the chaperone and the amyloid-forming protein may overlap in SEC, and the monomeric protein bands will also appear similar by SDS-PAGE, which may make it difficult to conclude whether there is an interaction or not. On the other hand, the presence of large aggregates or fibrils in the protein mixtures will tend to clog and damage the SEC column, therefore this technique is limited to native proteins and small

oligomers. A number of chaperones exist in a range of oligomeric states, which can further complicate analysis by SEC.

As a specific example (Fig. 6), in our lab we used a Superdex 200 10/300 GL column on an AKTA FPLC system to analyze the interaction between Hsp40 and Ure2 [33]. As the monomeric molecular weights of Hsp40 and Ure2 are quite similar (\approx 40 kDa), and both proteins are dimeric in solution, we chose to study N-terminally-truncated mutants of Ure2 with molecular weights of 29–30 kDa (90Ure2 and 105Ure2), to avoid overlapping peaks (by SEC) or bands (by SDS-PAGE). Hsp40s and Ure2 were preincubated for 15 min before injection onto the column. We found that the peaks of Ure2 and Ydj1 (the yeast homologue of Hsp40 that was found to inhibit fibril formation of Ure2 in solution) showed a significant shift when injected as a mixture. In contrast, when Ure2 was mixed with the human Hsp40 homologue Hdj2 (which showed no inhibition of Ure2 fibril formation) then no shift in the peaks was observed.

SEC has also been used to detect pre-fibrillar aggregates that form at the early stages of fibril formation. Lansbury and colleagues used a Superdex 200 column and found that α -synuclein prefibrils were eluted in the void peak, separate from monomers and fibrils [42,43]. Dedmon et al. found an interaction between Hsp70 and α -synuclein prefibrils using SEC [44]. They preincubated samples consisting of prefibrils and Hsp70, either alone or

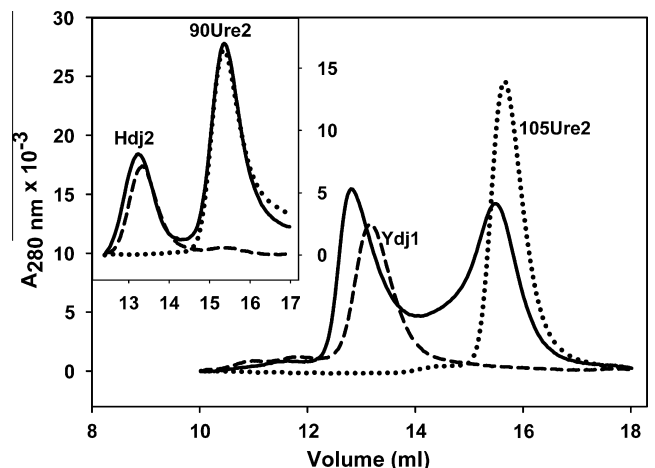


Fig. 6. SEC analysis of interactions between N-terminally truncated Ure2 and Ydj1 or Hdj2. The figure was reproduced from [33] with permission from the publisher.

together, at room temperature for 2 h and then centrifuged and filtered the samples to remove insoluble materials. The supernatants were injected onto a Superdex 200 10/30 GL column. Injections were performed in triplicate from independently prepared samples. The void peak area increased while the monomeric Hsp70 peak area decreased, signifying the co-elution of the two proteins. They used SDS–PAGE to analyze the void peak fraction, and a band corresponding to Hsp70 was observed only in the mixture, confirming their hypothesis that Hsp70 is involved in the formation of pre-fibrillar structures in the early stages of the α -synuclein fibril aggregation process. Huang et al. also detected an interaction between mutants of Hsp70 and α -synuclein prefibrils [35]. They found co-elution of α -synuclein with intact Hsp70 and a series of its domain-deletion mutants, thus identifying the domains of Hsp70 that are important for the interaction with prefibrils of α -synuclein.

3.2. Dynamic light scattering

The diffusion of particles in solution induces fluctuations in the intensity of scattered light [45]. Dynamic light scattering (DLS) detects these fluctuations using an autocorrelator on a microsecond time scale and is used to analyze the distribution of molecules and supramolecular aggregates as it is very sensitive to particle size [46]. Different sizes of molecules in the solution can be observed as different peaks, provided that their sizes differ sufficiently, and this technique is routinely used to determine the homogeneity of protein solutions, such as for use in protein crystallization trials.

DLS has been applied in studies of amyloid fibrillation [47]. Using this technique Liu et al. could observe peaks of insulin monomers, oligomers, and protofibrils [48]. Kusumoto et al. [49] and Tomski et al. [50] investigated the self-association kinetics of amyloid- β (A β) protein by DLS. With regard to chaperones, DLS can be used to investigate their interaction with amyloid-forming proteins. If a chaperone interacts with an amyloid-forming protein and forms oligomeric intermediates, the size of the intermediates can be determined precisely and will differ from that of the individual proteins.

3.3. Pull-down assays

Pull-down assays are used to detect interactions between chaperones and amyloid intermediate species or fibril seeds. The strategy for pull-down assays is based on the large molecular weight of fibril seeds. During ultracentrifugation in 40% sucrose, seeds will deposit in the pellet while native proteins, such as chaperones, will not. If the chaperone is found in the pellet, due to co-sedimentation of the chaperone and fibril seeds, this then indicates an interaction between the chaperone and the fibril seed. The steps involved in this method are outlined below.

3.3.1. Generation of fibril seeds

Mature fibrils are centrifuged and sonicated to form seeds. The sonication time and energy required is dependent on the amyloid-forming protein used, since the efficiency of sonication varies with the protein. Typically, the sonication time is several minutes. It is important to check the morphology of the seeds under AFM or EM to confirm optimal conditions. In our lab, a typical procedure to produce seeds of Ure2 involves collection of Ure2 fibrils by centrifugation (18000 g, 30 min), then after washing twice with buffer and resuspending, sonication is used to generate seeds [17].

3.3.2. Measurement of the concentration of protein in fibrillar form

The concentration of protein present in fibrils or seeds can be determined using one of a number of methods, as outlined below:

3.3.2.1. Dissolution of fibrils. One method used in our lab involves taking an aliquot of resuspended fibrils, adding SDS to a final concentration of 2% and then boiling for 5 min until the solution becomes clear, allowing the monomeric protein concentration to be measured by UV absorption or the BCA assay [17]. In another example, Yagi et al. solubilized seeds of α -synuclein with 6 N NaOH and determined the concentration of monomer generated from the seeds using a Bio-Rad protein assay kit [51]. The main concern with this method is whether or not the fibrils can be fully resolubilised, to allow accurate protein concentration determination in solution.

3.3.2.2. Measurement of soluble protein concentration and subtraction from initial monomer concentration. The monomeric protein concentration in the fibril supernatant can be measured and subtracted from the initial protein concentration, to estimate the concentration of protein in fibrillar form. In the case of α -synuclein, Huang et al. sonicated plateau-phase fibrils and concluded that all monomeric α -synuclein protein had formed fibrils by the plateau phase [35]. The concentration of monomeric protein contained in the seeds was thus the same as the monomer concentration of the original sample, before fibril formation commenced.

3.3.3. Performance of the pull-down assay

After incubation of seeds and chaperones under suitable conditions (typically for several hours), the samples are placed on a cushion of 40% sucrose followed by ultracentrifugation for 30 min, typically using a force of around 200,000 g. The pellets are then resuspended and analysed by SDS–PAGE. Like SEC, this technique requires that there is sufficiently strong affinity between the chaperone and the fibril seeds to observe an interaction. In contrast to the SEC technique, the pull-down assay allows observation of an interaction between the chaperone and relatively large fibrillar aggregates, rather than just between the native states or small oligomers.

3.4. Surface plasmon resonance

Surface plasmon resonance (SPR) is a well-established technique, widely used for studying the affinity of interactions between biological molecules such as proteins, nucleic acids, lipids and small molecules [52]. Indeed, a particular advantage of the SPR technique is that it readily detects interactions not only between molecules of similar size, but also, for example, interactions between proteins and small-molecule drugs, substrates, or cofactors. SPR has been applied in a wide range of areas, such as food safety and quality control, medical diagnostics, drug discovery, and environment protection [53–55].

Surface plasmons are surface electromagnetic waves that propagate in a direction parallel to the metal surface along a metal-dielectric interface. These waves are sensitive to changes of refractive index at this interface, such as the absorption of molecules to the metal surface. The plasmons are excited by the evanescent field of a laser that is shining onto the substrate. At a particular resonant angle, almost no light is reflected, as all the energy of the light beam is used to excite the plasmons. As the refractive index of the surface bound layer changes, so does the resonant angle. This shift in angle is detected. SPR requires one species to be immobilized on the sensor and the other to flow across the surface of the sensor. Surface plasmon resonance is observed when the immobilized species recognizes and captures the species in the mobile phase and leads to a local increase in the refractive index at the metal surface, causing an increase in the propagation constant of the surface plasmon wave propagating along the metal surface. The propagation constant can be measured accurately, generating a real-time signal [55,56]. SPR is thus a label-free

technique. Commercial SPR instruments are available from various companies and the Biacore™ system is the most frequently reported [56]. There are several methods for immobilizing a component on the sensor surface, the most typical being via covalent immobilization via free amines or thiols, or noncovalent immobilization via high-affinity interactions [57]. Recently, a toolbox of methods has been reported that allows covalent attachment of amyloid fibrils made from different proteins under varying solution conditions to gold coated biosensors [58], a strategy applicable to SPR. The review by Rich et al. provides a thorough introduction to SPR methods which can be employed for improving experimental design in order to optimize curve fitting for obtaining kinetic binding data, such as minimizing the amount of ligand immobilized, using high flow rates, carefully regenerating the sensor after use, and subtracting data from a reference surface or blank injection [59].

SPR has been used for monitoring the growth of amyloid fibrils [60–62], the binding of small ligands to amyloid structures [63,64], and the interaction between lipid and amyloid proteins [65–67]. It has also been used to investigate the interaction between chaperones and amyloid-forming proteins. Our group used SPR analysis to investigate the interaction between Hsp40 (Ydj1) and Ure2 [33]. Ure2 was immobilized onto a chip via amine coupling. We then obtained typical curves for binding and dissociation after applying the Ydj1 solution to the Ure2 chip (Fig. 7). Several models are available for fitting SPR curves; the appropriate choice of model will depend on the properties of the protein under study. In this case, the simplest model that gave a good fit to the data was a bivalent analyte model, which is consistent with the fact that both Ure2 and Ydj1 are dimers in the native state. As a control, we also performed a competitive binding experiment by mixing a 15-fold excess of

Ure2 in the mobile phase with Ydj1. The presence of Ure2 together with Ydj1 in solution was found to significantly reduce binding of Ydj1 to immobilized Ure2, confirming that the interaction between Ydj1 and Ure2 also occurs in solution [33]. As another example, Wilhelmus et al. studied the interaction of small heat shock proteins (sHsps) with A β protein using SPR and found that sHsps interact with some mutants of A β [68].

3.5. Quartz crystal microbalance (QCM)

Another approach for detecting protein–protein interactions that does not rely on a label is QCM, which is based on the high sensitivity of the resonant frequency of an oscillating quartz crystal to changes in its mass. The surface of the sensor is on an anti-node of the shear waves of the resonance frequency, and when the mass of the sensor increases, the frequency will decrease accordingly [62,69]. In the limit of small changes, these parameters have a linear relationship such that the change in mass on the sensor surface can be calculated by monitoring the change in resonance frequency [69]. In addition, the simultaneous monitoring of the energy dissipation of the oscillating crystal enables insight to be gained into the viscoelastic properties of the surface bound material [70].

Development of QCM has enabled the detection of liquid–solid interface phenomena. QCM has been applied to studies of biochemical processes, biomimetic systems, biosensors and drug discovery [71]. Recently, QCM has been used to study amyloid growth of a number of proteins, such as insulin, A β , α -synuclein and lysozyme, including in the presence of chaperones, macromolecular crowding agents or small molecules [62,69,72,73]. One advantage of using QCM is that it does not require as much protein or time as traditional fluorescence-binding methods. A mass increase of

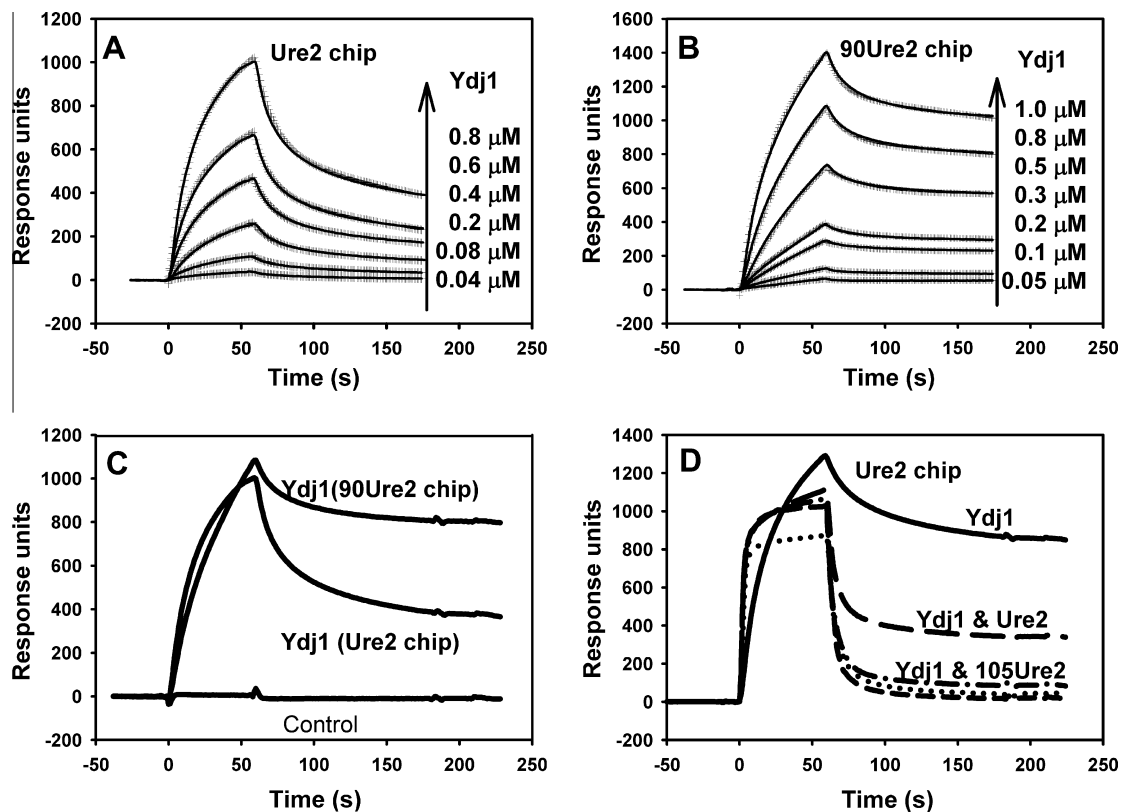


Fig. 7. Interaction of Ydj1 with Ure2 or N-terminally truncated Ure2, monitored by SPR. Ydj1 was in the mobile phase and Ure2 and its mutants were immobilized on the chips. The figure was reproduced from [33] with permission from the publisher. (A, B) Binding of Ydj1 to Ure2 or 90Ure2 increased with increasing Ydj1 concentration. (C) Comparison of binding to immobilized Ure2 and 90Ure2. As a control, Hsp104 showed zero binding to the Ure2 chip under the same conditions. (D) Binding of Ydj1 to immobilized Ure2 in the presence or absence of a competing 15-fold excess of Ure2 or 105Ure2 in the mobile phase.

only 30 pg corresponds to a 1 Hz decrease on a 4.9 mm² electrode [74]. It has also been reported that QCM is capable of detecting fibril elongation at monomer concentrations as low as 500 nM [75]. While measurements can be completed within several minutes or hours rather than several days, QCM still offers real-time data in a similar way to fluorescence-binding assays, making it a convenient method for monitoring fibril formation [74].

The first step in QCM is to immobilize fibril seeds (which are generated by sonicating mature fibrils) onto the surface of the sensor and passivate the rest of the surface, which is important to avoid non-specific binding [69]. Irreversible covalent attachment is an important requirement, as one of the main advantages of QCM relies on the fact that the growth of a constant ensemble of fibrils can be monitored under varying conditions, such that the measured rates can be directly compared. Methods have been developed to achieve this attachment also for amyloidogenic proteins that do not contain cysteine residues [58]. These methods rely on a minimally invasive attachment of small sulfur containing molecules, that enable a strong and irreversible attachment of the amyloid seed fibrils onto the QCM electrode.

As fresh protein solution is pumped onto the surface of the sensor, the fibrils will elongate leading to an increase in the mass of the sensor and a corresponding decrease in the resonant frequency. If the presence of chaperone in the fluid phase results in a slower increase in mass than the amyloid-generating protein alone, this suggests the interaction of chaperone with the amyloid-generating protein or seeds.

As an example of the application of the QCM technique (Fig. 8), Knowles et al. observed a slower increase in sensor mass while applying a synthetic chaperone or sHsp in an insulin solution [69]. After analyzing the rate of insulin amyloid formation, they concluded that the synthetic chaperone interacted with insulin monomers while sHsp bound to the growth site on the sensor. In another study, Okuno et al. designed several new compounds as candidate inhibitors of A β aggregation [72], and used QCM to evaluate their inhibitory effects. A slight frequency drop occurred as the inhibitor was added, but then the frequency increased again after 2.5 h. They concluded that the inhibitor interacted with the A β aggregates and then induced their dissolution from the sensor.

4. Concluding remarks

Chaperones, as components of the protein quality control system, are considered to play a crucial role in controlling protein misfolding and amyloid formation *in vivo*. Here we have discussed some of the methods that are commonly used for analyzing interaction mechanisms. The best choice of experimental conditions will vary according to the properties of the protein under investigation, and it is important to first optimize conditions in order to obtain reproducible and meaningful results. Fluorescence-binding assays and filter retardation assays are commonly carried out to detect the effect of chaperones on the time course of amyloid fibril formation, and are generally combined with morphological observations by EM or AFM. These well-established techniques, combined with recent developments in the application of biosensor assays, are contributing to our understanding of the underlying mechanisms by which chaperones can influence amyloid fibril formation.

Acknowledgements

Work in the Perrett lab is supported by grants from the National Natural Science Foundation of China (30870482, 31070656, 31000342), the Chinese Ministry of Science and Technology (2006CB500703, 2006CB910903), the Chinese Academy of Sciences (KSCX2-YW-R-119, KSCX2-YW-R-256), and a Royal Society International Joint-Project Grant (to Laura Itzhaki and S.P.). We thank Alex Buell, Joy Fleming and Gary Jones for comments on the manuscript.

References

- [1] F. Chiti, C.M. Dobson, Protein misfolding, functional amyloid, and human disease, *Annu. Rev. Biochem.* 75 (2006) 333–366.
- [2] S. Perrett, G.W. Jones, Insights into the mechanism of prion propagation, *Curr. Opin. Struct. Biol.* 18 (2008) 52–59.
- [3] J.D. Sipe, A.S. Cohen, Review: history of the amyloid fibril, *J. Struct. Biol.* 130 (2000) 88–98.
- [4] M.R. Nilsson, Techniques to study amyloid fibril formation *in vitro*, *Methods* 34 (2004) 151–160.
- [5] T.R. Jahn, O.S. Makin, K.L. Morris, K.E. Marshall, P. Tian, P. Sikorski, L.C. Serpell, The common architecture of cross-beta amyloid, *J. Mol. Biol.* 395 (2010) 717–727.
- [6] Y. Jiang, H. Li, L. Zhu, J.M. Zhou, S. Perrett, Amyloid nucleation and hierarchical assembly of Ure2p fibrils. Role of asparagine/glutamine repeat and nonrepeat regions of the prion domains, *J. Biol. Chem.* 279 (2004) 3361–3369.
- [7] F.U. Hartl, M. Hayer-Hartl, Molecular chaperones in the cytosol: from nascent chain to folded protein, *Science* 295 (2002) 1852–1858.
- [8] J.R. Glover, S. Lindquist, Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins, *Cell* 94 (1998) 73–82.

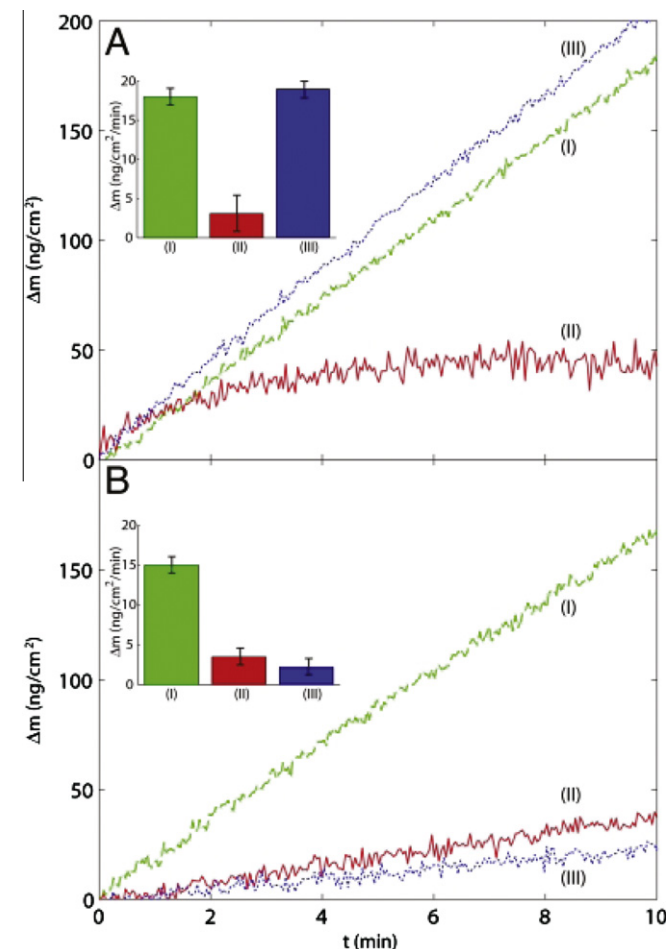


Fig. 8. Inhibition of amyloid growth by a chemical chaperone and a sHsp. The figure was reproduced from [69] with permission, copyright (2007) National Academy of Sciences, USA. (A) Insulin fibrils were first exposed to an insulin solution (I), the solution was then replaced by a mixture containing the same amount of insulin but in addition 1 M TMAO (II), and finally a solution of insulin in buffer alone (III). (Inset) The growth rates observed during the successive stages are shown. (B) Insulin fibrils were first exposed to an insulin solution (I), the solution was then replaced by a mixture of 0.5:1 M ratio of sHsp to insulin (II), and finally a solution of insulin in buffer alone (III). Kinetic data are shown starting from 120 s after the injection of the solutions.

- [9] S.M. Doyle, S. Wickner, Hsp104 and ClpB: protein disaggregating machines, *Trends Biochem. Sci.* 34 (2009) 40–48.
- [10] P.J. Muchowski, J.L. Wacker, Modulation of neurodegeneration by molecular chaperones, *Nat. Rev. Neurosci.* 6 (2005) 11–22.
- [11] W.E. Balch, R.I. Morimoto, A. Dillin, J.W. Kelly, Adapting proteostasis for disease intervention, *Science* 319 (2008) 916–919.
- [12] G.W. Jones, M.F. Tuite, Chaperoning prions: the cellular machinery for propagating an infectious protein?, *Bioessays* 27 (2005) 823–832.
- [13] A.T. Petkova, R.D. Leapman, Z. Guo, W.M. Yau, M.P. Mattson, R. Tycko, Self-propagating molecular-level polymorphism in Alzheimer's beta-amyloid fibrils, *Science* 307 (2005) 262–265.
- [14] L. Zhu, X.J. Zhang, L.Y. Wang, J.M. Zhou, S. Perrett, Relationship between stability of folding intermediates and amyloid formation for the yeast protein Ure2p: a quantitative analysis of the effects of pH and buffer system, *J. Mol. Biol.* 328 (2003) 235–254.
- [15] P. Lopez-Buesa, C. Pfund, E.A. Craig, The biochemical properties of the ATPase activity of a 70-kDa heat shock protein (Hsp70) are governed by the C-terminal domains, *Proc. Natl. Acad. Sci. U.S.A.* 95 (1998) 15253–15258.
- [16] J. Brange, L. Langkjoer, Insulin structure and stability, *Pharm. Biotechnol.* 5 (1993) 315–350.
- [17] L. Fei, S. Perrett, Disulfide bond formation significantly accelerates the assembly of Ure2p fibrils because of the proximity of a potential amyloid stretch, *J. Biol. Chem.* 284 (2009) 11134–11141.
- [18] B. Bosl, V. Grimminger, S. Walter, Substrate binding to the molecular chaperone Hsp104 and its regulation by nucleotides, *J. Biol. Chem.* 280 (2005) 38170–38176.
- [19] B. Gao, L. Greene, E. Eisenberg, Characterization of nucleotide-free uncoating ATPase and its binding to ATP, ADP, and ATP analogues, *Biochemistry* 33 (1994) 2048–2054.
- [20] S.M. Doyle, J. Shorter, M. Zolkiewski, J.R. Hoskins, S. Lindquist, S. Wickner, Asymmetric deceleration of ClpB or Hsp104 ATPase activity unleashes protein-remodeling activity, *Nat. Struct. Mol. Biol.* 14 (2007) 114–122.
- [21] B. Frost, J. Ollesch, H. Wille, M.I. Diamond, Conformational diversity of wild-type Tau fibrils specified by templated conformation change, *J. Biol. Chem.* 284 (2009) 3546–3551.
- [22] M. Yonetani, T. Nonaka, M. Masuda, Y. Inukai, T. Oikawa, S. Hisanaga, M. Hasegawa, Conversion of wild-type alpha-synuclein into mutant-type fibrils and its propagation in the presence of A30P mutant, *J. Biol. Chem.* 284 (2009) 7940–7950.
- [23] P.S. Vassar, C.F. Culling, Fibrosis of the breast, *AMA Arch Pathol* 67 (1959) 128–133.
- [24] H. LeVine 3rd, Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution, *Protein Sci.* 2 (1993) 404–410.
- [25] R. Eisert, L. Felau, L.R. Brown, Methods for enhancing the accuracy and reproducibility of Congo red and thioflavin T assays, *Anal. Biochem.* 353 (2006) 144–146.
- [26] H. LeVine 3rd, Quantification of beta-sheet amyloid fibril structures with thioflavin T, *Methods Enzymol.* 309 (1999) 274–284.
- [27] X. Ma, L. Liu, X. Mao, L. Niu, K. Deng, W. Wu, Y. Li, Y. Yang, C. Wang, Amyloid beta (1–42) folding multiplicity and single-molecule binding behavior studied with STM, *J. Mol. Biol.* 388 (2009) 894–901.
- [28] M. Groenning, Binding mode of Thioflavin T and other molecular probes in the context of amyloid fibrils—current status, *J. Chem. Biol.* 3 (2010) 1–18.
- [29] S. Chen, V. Berthelie, J.B. Hamilton, B. O'Nuallain, R. Wetzel, Amyloid-like features of polyglutamine aggregates and their assembly kinetics, *Biochemistry* 41 (2002) 7391–7399.
- [30] R. Khurana, C. Coleman, C. Ionescu-Zanetti, S.A. Carter, V. Krishna, R.K. Grover, R. Roy, S. Singh, Mechanism of thioflavin T binding to amyloid fibrils, *J. Struct. Biol.* 151 (2005) 229–238.
- [31] R. Sabate, S.J. Saupé, Thioflavin T fluorescence anisotropy: an alternative technique for the study of amyloid aggregation, *Biochem. Biophys. Res. Commun.* 360 (2007) 135–138.
- [32] J. Wall, C.L. Murphy, A. Solomon, In vitro immunoglobulin light chain fibrillogenesis, *Methods Enzymol.* 309 (1999) 204–217.
- [33] H.Y. Lian, H. Zhang, Z.R. Zhang, H.M. Loovers, G.W. Jones, P.J. Rowling, L.S. Itzhaki, J.M. Zhou, S. Perrett, Hsp40 interacts directly with the native state of the yeast prion protein Ure2 and inhibits formation of amyloid-like fibrils, *J. Biol. Chem.* 282 (2007) 11931–11940.
- [34] H. Zhang, H.M. Loovers, L.Q. Xu, M. Wang, P.J. Rowling, L.S. Itzhaki, W. Gong, J.M. Zhou, G.W. Jones, S. Perrett, Alcohol oxidase (AOX1) from *Pichia pastoris* is a novel inhibitor of prion propagation and a potential ATPase, *Mol. Microbiol.* 71 (2009) 702–716.
- [35] C. Huang, H. Cheng, S. Hao, H. Zhou, X. Zhang, J. Gao, Q.H. Sun, H. Hu, C.C. Wang, Heat shock protein 70 inhibits alpha-synuclein fibril formation via interactions with diverse intermediates, *J. Mol. Biol.* 364 (2006) 323–336.
- [36] S.W. Davies, K. Sathasivam, C. Hobbs, P. Doherty, L. Mangiarini, E. Scherzinger, E.E. Wanker, G.P. Bates, Detection of polyglutamine aggregation in mouse models, *Methods Enzymol.* 309 (1999) 687–701.
- [37] E. Scherzinger, A. Sittler, K. Schweiger, V. Heiser, R. Lurz, R. Hasenbank, G.P. Bates, H. Lehrach, E.E. Wanker, Self-assembly of polyglutamine-containing huntingtin fragments into amyloid-like fibrils: implications for Huntington's disease pathology, *Proc. Natl. Acad. Sci. U.S.A.* 96 (1999) 4604–4609.
- [38] P.J. Muchowski, G. Schaffar, A. Sittler, E.E. Wanker, M.K. Hayer-Hartl, F.U. Hartl, Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils, *Proc. Natl. Acad. Sci. U.S.A.* 97 (2000) 7841–7846.
- [39] A. Sittler, R. Lurz, G. Lueder, J. Priller, H. Lehrach, M.K. Hayer-Hartl, F.U. Hartl, E.E. Wanker, Geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a cell culture model of Huntington's disease, *Hum. Mol. Genet.* 10 (2001) 1307–1315.
- [40] H.A. Krol, P.M. Krawczyk, K.S. Bosch, J.A. Aten, E.M. Hol, E.A. Reits, Polyglutamine expansion accelerates the dynamics of ataxin-1 and does not result in aggregate formation, *PLoS One* 3 (2008) e1503.
- [41] A. Boeddrich, R. Lurz, E.E. Wanker, Huntingtin fragments form aggresome-like inclusion bodies in mammalian cells, *Methods Mol. Biol.* 232 (2003) 217–229.
- [42] J.D. Harper, S.S. Wong, C.M. Lieber, P.T. Lansbury Jr., Assembly of A beta amyloid protofibrils: an in vitro model for a possible early event in Alzheimer's disease, *Biochemistry* 38 (1999) 8972–8980.
- [43] K.A. Conway, S.J. Lee, J.C. Rochet, T.T. Ding, R.E. Williamson, P.T. Lansbury Jr., Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy, *Proc. Natl. Acad. Sci. U.S.A.* 97 (2000) 571–656.
- [44] M.M. Dedmon, J. Christodoulou, M.R. Wilson, C.M. Dobson, Heat shock protein 70 inhibits alpha-synuclein fibril formation via preferential binding to prefibrillar species, *J. Biol. Chem.* 280 (2005) 14733–14740.
- [45] R.M. Murphy, M.M. Pallitto, Probing the kinetics of beta-amyloid self-association, *J. Struct. Biol.* 130 (2000) 109–122.
- [46] M.M. Domingues, P.S. Santiago, M.A. Castanho, N.C. Santos, What can light scattering spectroscopy do for membrane-active peptide studies?, *J. Pept. Sci.* 14 (2008) 394–400.
- [47] A. Ahmad, V.N. Uversky, D. Hong, A.L. Fink, Early events in the fibrillation of monomeric insulin, *J. Biol. Chem.* 280 (2005) 42669–42675.
- [48] R. Liu, M. He, R. Su, Y. Yu, W. Qi, Z. He, Insulin amyloid fibrillation studied by terahertz spectroscopy and other biophysical methods, *Biochem. Biophys. Res. Commun.* 391 (2010) 862–867.
- [49] Y. Kusumoto, A. Lomakin, D.B. Teplow, G.B. Benedek, Temperature dependence of amyloid beta-protein fibrillization, *Proc. Natl. Acad. Sci. U.S.A.* 95 (1998) 12277–12282.
- [50] S.J. Tomski, R.M. Murphy, Kinetics of aggregation of synthetic beta-amyloid peptide, *Arch. Biochem. Biophys.* 294 (1992) 630–638.
- [51] H. Yagi, E. Kusaka, K. Hongo, T. Mizobata, Y. Kawata, Amyloid fibril formation of alpha-synuclein is accelerated by preformed amyloid seeds of other proteins: implications for the mechanism of transmissible conformational diseases, *J. Biol. Chem.* 280 (2005) 38609–38616.
- [52] M. Willander, S. Al-Hilli, Analysis of biomolecules using surface plasmons, *Methods Mol. Biol.* 544 (2009) 201–229.
- [53] M.A. Cooper, Optical biosensors in drug discovery, *Nat. Rev. Drug Discov.* 1 (2002) 515–528.
- [54] J. Homola, Surface plasmon resonance sensors for detection of chemical and biological species, *Chem. Rev.* 108 (2008) 462–493.
- [55] J. Homola, Present and future of surface plasmon resonance biosensors, *Anal. Bioanal. Chem.* 377 (2003) 528–539.
- [56] S. Scarano, M. Mascini, A.P. Turner, M. Minunni, Surface plasmon resonance imaging for affinity-based biosensors, *Biosens. Bioelectron.* 25 (2010) 957–966.
- [57] A.J. Kimple, R.E. Muller, D.P. Siderovski, F.S. Willard, A capture coupling method for the covalent immobilization of hexahistidine tagged proteins for surface plasmon resonance, *Methods Mol. Biol.* 627 (2010) 91–100.
- [58] A.K. Buell, D.A. White, M.E. Welland, T.P.J. Knowles, C.M. Dobson, Surface attachment of protein fibrils via covalent modification strategies, *J. Phys. Chem. B.* 114 (2010) 10925–10938.
- [59] R.L. Rich, D.G. Myszka, Advances in surface plasmon resonance biosensor analysis, *Curr. Opin. Biotechnol.* 11 (2000) 54–61.
- [60] M.J. Cannon, A.D. Williams, R. Wetzel, D.G. Myszka, Kinetic analysis of beta-amyloid fibril elongation, *Anal. Biochem.* 328 (2004) 67–75.
- [61] J. Ryu, H.A. Jung, M.G. Kim, C.B. Park, Surface plasmon resonance analysis of Alzheimer's beta-amyloid aggregation on a solid surface: from monomers to fully-grown fibrils, *Anal. Chem.* 80 (2008) 2400–2407.
- [62] D.A. White, A.K. Buell, C.M. Dobson, M.E. Welland, T.P. Knowles, Biosensor-based label-free assays of amyloid growth, *FEBS Lett* 583 (2009) 2587–2592.
- [63] G. Habicht, C. Haupt, R.P. Friedrich, P. Hortschansky, C. Sachse, J. Meinhardt, K. Wieligmann, G.P. Gellermann, M. Brodhun, J. Gotz, K.J. Halbhuber, C. Rocken, U. Horn, M. Fandrich, Directed selection of a conformational antibody domain that prevents mature amyloid fibril formation by stabilizing Abeta protofibrils, *Proc. Natl. Acad. Sci. U.S.A.* 104 (2007) 19232–19237.
- [64] S. Kawatake, Y. Nishimura, S. Sakaguchi, T. Iwaki, K. Doh-ura, Surface plasmon resonance analysis for the screening of anti-prion compounds, *Biol. Pharm. Bull.* 29 (2006) 927–932.
- [65] X. Hou, S.J. Richardson, M.I. Aguilar, D.H. Small, Binding of amyloidogenic transthyretin to the plasma membrane alters membrane fluidity and induces neurotoxicity, *Biochemistry* 44 (2005) 11618–11627.
- [66] M.S. Lin, H.M. Chiu, F.J. Fan, H.T. Tsai, S.S. Wang, Y. Chang, W.Y. Chen, Kinetics and enthalpy measurements of interaction between beta-amyloid and liposomes by surface plasmon resonance and isothermal titration microcalorimetry, *Colloids Surf. B Biointerfaces* 58 (2007) 231–236.
- [67] X. Hou, D.H. Small, M.I. Aguilar, Surface plasmon resonance spectroscopy in determination of the interactions between amyloid beta proteins (Abeta) and lipid membranes, *Methods Mol. Biol.* 627 (2010) 225–235.
- [68] M.M. Wilhelmus, W.C. Boelens, I. Otte-Holler, B. Kamps, R.M. de Waal, M.M. Verbeek, Small heat shock proteins inhibit amyloid-beta protein aggregation and cerebrovascular amyloid-beta protein toxicity, *Brain Res.* 1089 (2006) 67–78.

- [69] T.P. Knowles, W. Shu, G.L. Devlin, S. Meehan, S. Auer, C.M. Dobson, M.E. Welland, Kinetics and thermodynamics of amyloid formation from direct measurements of fluctuations in fibril mass, *Proc. Natl. Acad. Sci. U.S.A.* 104 (2007) 10016–10021.
- [70] M. Rodahl, F. Hook, C. Fredriksson, C.A. Keller, A. Krozer, P. Brzezinski, M. Voinova, B. Kasemo, Simultaneous frequency and dissipation factor QCM measurements of biomolecular adsorption and cell adhesion, *Faraday Discuss* (1997) 229–246.
- [71] K.A. Marx, Quartz crystal microbalance: a useful tool for studying thin polymer films and complex biomolecular systems at the solution-surface interface, *Biomacromolecules* 4 (2003) 1099–1120.
- [72] H. Okuno, K. Mori, T. Okada, Y. Yokoyama, H. Suzuki, Development of aggregation inhibitors for amyloid-beta peptides and their evaluation by quartz-crystal microbalance, *Chem. Biol. Drug Des.* 69 (2007) 356–361.
- [73] D.A. White, A.K. Buell, T.P. Knowles, M.E. Welland, C.M. Dobson, Protein aggregation in crowded environments, *J. Am. Chem. Soc.* 132 (2010) 5170–5175.
- [74] H. Okuno, K. Mori, T. Jitsukawa, H. Inoue, S. Chiba, Convenient method for monitoring Abeta aggregation by quartz-crystal microbalance, *Chem. Biol. Drug Des.* 68 (2006) 273–275.
- [75] J.A. Kotarek, K.C. Johnson, M.A. Moss, Quartz crystal microbalance analysis of growth kinetics for aggregation intermediates of the amyloid-beta protein, *Anal. Biochem.* 378 (2008) 15–24.