

Title: Diagnostics for amyloid fibril formation: where to begin.

Running Title: Testing for amyloid fibril formation

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i. Summary

25 proteins are known form amyloid fibrils *in vivo* in association with disease (1). However, the fundamental ability of a protein to form amyloid-like fibrils is far more widespread than in just the proteins associated with disease, and indeed this property can provide insight into the basic thermodynamics of folding and misfolding pathways. But how does one determine whether a protein has formed amyloid-like fibrils? In this chapter, we cover the basic steps towards defining the amyloid-like properties of a protein and how to measure the kinetics of fibrillization. We describe several basic tests for aggregation and the binding to two classic amyloid-reactive dyes, Congo Red and thioflavin T, which are key indicators to the presence of fibrils.

ii. Keywords

Protein misfolding, Congo Red, thioflavin T, aggregation, birefringence, amyloid, fibril, procedures, protocol, method

1. Introduction

When considering whether a protein is an “amyloid” or not, it is worth first considering the historical context of “amyloid”. Amyloid, derived from the latin word *amylum* for “starch”, was first described as iodine-reactive deposits in the brain (2). While the iodine reactivity likely reflects proteoglycan staining rather than starch, which is not present in human tissue, later studies described amyloid deposits as being red-green birefringent under polarized light in the presence of the stain Congo Red (3,4). *Ex vivo* amyloid deposits were shown to contain fibrous material, which confers the Congo Red birefringent properties observed under light microscopy (5). From these and other studies, the definition for “amyloid” evolved as a histological description for extracellular deposits exhibiting Congo Red red-green birefringence comprising primarily of one or more of 25 particular proteins (1).

Since the mid 1990s, much insight has been gleaned on the molecular structure and mechanisms of formation of amyloid fibrils (6). While the histological definition of “amyloid” does not directly translate to the biochemical and biophysical properties of the material, a number of structural attributes common to the proteins in amyloid deposits have been identified. These include an aggregate assembly state of fibrillar morphology rich in β -sheet secondary structure comprising β -strands aligned perpendicular to the long fibril axis (7-9). The amyloid structure for many proteins such as lysozyme, was shown to reflect a non-native alternative conformation to the native state, suggesting amyloid depicts the consequences of protein misfolding *in vivo* (10). Hence exploring

how environmental parameters or mutations mediate fibril formation can be useful for understanding the folding thermodynamics of a protein.

The biophysical and structural properties of amyloid proteins have more recently been shown to occur in a large number of proteins other than those classically defined by pathology, which has led to the suggestion that proteins are inherently capable of acquiring an amyloid-like structure under certain conditions (11). Indeed, some bacterial and fungal proteins seem to have evolved specifically as natural “amyloid” structure for normal function (12,13).

In this chapter we describe the first steps in defining an amyloid fibril from a biochemical perspective. We anticipate that the approaches described here be the starting point for experimentalists and feed directly to the other chapters of this book for a more detailed analysis. We cover three basic spectroscopic approaches: direct assessment of aggregation, and the binding to two classic amyloid dyes Congo Red and thioflavin T and finish with procedures for defining the kinetics of amyloid-fibril formation.

2. Materials

2.1 Measuring aggregation state by centrifugation

1. Microcentrifuge.
2. A high speed benchtop centrifuge (see **Note 1**)
3. Sample buffer, eg phosphate buffered saline (See **Note 2**).
4. UV-Vis Spectrophotometer.
5. Protein detection reagents (See **Note 3**)

2.2 Measuring aggregation state by gel filtration chromatography

1. HPLC system/peristaltic pump (if available) and chromatography column (eg Superdex 200 or Sepharose CL-4B from GE Biosciences).
2. Sample buffer, eg phosphate buffered saline. When using HPLC systems, all buffers must be filtered through a 0.22 μm filter paper to remove particulates. Always prepare fresh buffers to prevent mould contamination (Refer also to **Note 2**)

2.3 Measuring Congo Red reactivity and birefringence

1. Congo Red for the spectroscopic assay. Prepare a 1 mM solution in ethanol (Congo Red is poorly soluble in water).
2. Congo Red for the birefringence assay. Add a saturating amount of Congo Red to a small volume of 80% (v/v) ethanol, 20% (v/v) distilled water, saturated NaCl. Stir the solution for 10 minutes and allow the undissolved Congo Red to settle. Filter the supernatant through a 0.2 μm syringe filter to obtain the working solution. This solution can be stored for a number of days if kept in the dark but should be filtered directly before use.
3. Microfiltration centrifugation device. Any of the standard brands is fine. It is best to use a high nominal molecular weight cut-off (NMWCO), which facilitates rapid concentration without loss of fibrillar material relative to the low NMWCO.
4. Light microscope equipped with incident light and objective polarisers (for example, those commonly used to examine protein crystals).
5. A spectrophotometer capable of wavelength scans in the wavelength range 300-700 nm.

6. Sample buffer, eg phosphate buffered saline (Refer also to **Note 2**)

2.4 Measuring thioflavin T reactivity

1. Prepare a 1 mM stock thioflavin T solution by dissolving the appropriate amount of dye in phosphate buffered saline, and filter the solution through a 0.22 μm syringe filter. ThT solutions must be stored in the dark and are stable at room temperature under these conditions for at least one week. Solutions can also be stored long term at $-20\text{ }^{\circ}\text{C}$ in the dark for a number of months.
2. Fluorimeter. Any standard instrument is fine. A plate reader format is most useful for kinetic studies.
3. Sample buffer, eg phosphate buffered saline (Refer also to **Note 2**)

2.5 Kinetic assays

1. Refer to materials and buffers needed sections 2.1-2.4.
2. Mineral oil.

3. Methods

3.1 Measuring aggregation state by centrifugation

Amyloid fibrils are characterized by their large molecular mass relative to the “normal” form of the protein. Hence centrifugation remains a simple and direct test for the solubility of the protein. Often large fibrils appear flocculent in solution when viewed through a microfuge tube against bright light. Such fibrils are easily pelleted in a microcentrifuge and can be readily measured for solubility as follows:

1. Prepare two parallel samples of the protein in two 1.5 ml microcentrifuge tubes. At least 200 μ l of sample is needed in one of the tubes for centrifugation, and 60 μ l of sample is needed in the second tube for the non-centrifuged sample.
2. For the 200 μ l sample, centrifuge for 30 min at room temperature at maximum speed in a microcentrifuge. See **Note 4**.
3. Carefully remove and keep aside 50 μ l of the supernatant without disturbing the pellet.
4. Measure the protein concentration of the supernatant versus the uncentrifuged sample. It is important to thoroughly mix the uncentrifuged sample before analysis to ensure that the aggregates have not settled. The protein detection can be done a number of ways and adapted to a 96-well plate for use in a plate reader. (See **Note 3**)

It is important to note that not all fibrils (for example those formed by apoC-II (14) or apoE (15)) are visibly flocculent or pellet in a microcentrifuge. In addition, small oligomer precursors to large fibrils may also remain in solution. Hence, to comprehensively capture the oligomeric, aggregated forms of the protein, pelleting should also be investigated using a high-speed benchtop centrifuge (such as a Beckman Optima TLX ultracentrifuge).

5. Prepare aliquots of samples as described above in step 1.
6. Centrifuge the 200 μ l aliquot at 100,000 g for 30 min using a high speed fixed angle benchtop centrifuge (for example, the Beckman Coulter TL120.2 rotor with clear polycarbonate tubes (cat# 343778 from Beckman Coulter). See **Note 4**.

7. The supernatant should be assessed as described above in step 4.

3.2 Measuring aggregation state by gel filtration chromatography

Another assay for measuring aggregation is gel filtration chromatography. Gel-filtration chromatography separates molecules on the basis of the ability of a solute, such as a protein, to diffuse into and out of the stationary-phase relative to the rate of the bulk flow of the mobile-phase. In general, the rates of diffusion into the stationary-phase depend on the size and shape of the solute, the composition of the stationary-phase, and the temperature and buffer/solvent conditions. In practise, this means that small, rapidly diffusing molecules, such as the native protein (typically less than 100 kDa) move slowly through a gel-filtration column, while larger particles, such as fibrils, are either partially or totally excluded from the stationary phase and hence move rapidly.

Amyloid-like fibrils tend to elute in the void volume of the most common resins and very large fibrils can be occluded from entering the column altogether– and this needs to be considered when using expensive pre-packed columns, which can become irreversibly blocked. However, many smaller fibrils can be effectively separated on a column and this provides a convenient means to measure the proportion of for example, monomers from aggregates, which typically elute in the void volume (Fig 1).

An inexpensive option is to pack a column with a low-cost resin such as Sepharose CL-2B, which is a cross-linked agarose resin from GE Healthcare, and to elute the proteins by a peristaltic pump or gravity flow. This resin has a nominal fractionation range of approximately 70,000 to 4,000,000 Da for proteins and has a high flow rate capacity making it ideal for separating proteins that have a very large difference in size, such as fibrils from the unaggregated counterpart.

1. Pack a small column (eg 1 × 30 cm Econo-column from BioRad) with Sepharose CL-2B and equilibrate in buffer of choice. See **Notes 5 and 6**.
2. Run the column with several column volumes of buffer to ensure the resin is fully settled (see **Notes 7 and 8**).
3. Load samples containing the protein carefully on the top of the column in no more than 0.5 ml for a 1 × 30 column (a good rule of thumb is to load no more than 0.02 column volumes of sample to reduce peak broadening).
4. Elute proteins by gravity flow once all the sample has entered the gel, ensuring that the top of the column does not dry out (refer to **Note 5**). Alternatively use a peristaltic pump running at approximately 0.5 ml/min to apply the sample followed by the buffer. It is useful to also run sizing standards to determine the void and total volumes of the column (See **Note 9**).
5. Collect 0.2-0.5 ml fractions and measure the protein concentration by either $A_{280\text{nm}}$ or by a protein detection kit (refer to **Note 3**). It is often convenient to elute 0.25 ml fractions directly into a 96-well plate for rapid screening in a platereader.

3.3 Measuring Congo Red reactivity and birefringence

While Congo Red is the classic histological stain for detecting amyloid *in vivo* it can also be used *in vitro* to test for amyloid fibrils (16). Upon binding to amyloid, the optical properties of Congo Red are altered which can be detected using two methods. The first method follows changes to the absorption spectrum of Congo Red when bound to fibrils.

The second, detects the red-green birefringence exhibited by fibril-bound Congo Red under cross-polarised light.

3.3.1 *Method 1: Congo Red spectroscopic assay*

1. Prepare a fresh solution of 100 μM Congo Red in phosphate buffered saline (from the 1 mM stock in ethanol) and filter through a 0.22 μm syringe filter immediately prior to use to remove particulates and undissolved Congo Red.
2. Prepare test samples by mixing together the protein sample with the Congo Red solution. Ensure that the final Congo Red concentration is between 2 – 20 μM . Also the protein concentration (the concentration of monomeric protein) in the sample should generally not exceed 100 μM . See **Note 10**.
3. Incubate for 5 mins at room temperature.
4. Prepare control samples. Control A is Congo Red alone at the same concentration and final buffer composition as that in step 2. This control defines the Congo Red reference spectrum. It is important to dilute Congo Red into the same buffer as which the protein has been prepared in. Control B is the protein alone at the same concentration as the test samples, and also the same buffer composition (ie dilute the protein into phosphate buffered saline instead of the Congo Red solution). Control B enables a correction to be made to account for solution turbidity caused by protein aggregates.
5. Set a UV/Vis spectrophotometer to acquire absorbance spectra between 300 – 700 nm and zero the instrument against phosphate buffered saline. Measure the absorbance spectra of all test and control samples. At this point a shoulder peak

centered around 540 nm, which is indicative of amyloid fibrils, may be apparent in the test sample that is not visible in control sample A.

6. Subtract the spectrum of control B (protein alone) from the test spectrum to correct for turbidity. From the resultant spectrum, subtract the spectrum acquired for control A (Congo Red alone). A peak in the resulting final difference spectrum at 540 nm is generally considered indicative of amyloid fibrils. See **Note 11**.

3.3.2 Method 2: Congo Red birefringence assay

There are various ways in which protein aggregates can be prepared for the birefringent assay. All of them in essence condense Congo Red labeled aggregates to masses large enough to be visualized by microscopy. The different options for preparation should be trialled for different proteins.

3.3.2.1 Preparation option A

1. Place 10-20 μ l of aggregate suspension on a poly-lysine coated microscope slide and allow to air dry at room temperature or in a 37 °C incubator.
2. Place ~50 μ l of stain onto the dried protein sample and incubate for a few minutes.
3. Blot away excess solution and allow to air dry once more.
4. If necessary, excess stain can be removed by washing the sample with a small volume of distilled water.

3.3.2.2 Preparation option B

1. Pellet 1 ml volume of aggregates by centrifugation and remove the supernatant.

2. Resuspend the aggregate pellet in Congo Red stain solution and incubate for 5 minutes.
3. Pellet the stained fibrils once more and resuspend in a small volume of distilled water. See **Note 12**.
4. Place the stained aggregate suspension on a poly-lysine coated microscope slide and allow to air dry at room temperature or in a 37 °C incubator.

3.3.2.3 *Preparation option C* (see **Note 13**)

1. Place protein solution containing aggregates in a centrifugation filtration device and concentrate proteins until large (> mm in size) clumps of protein appear.
2. Collect aggregates and resuspend in Congo Red stain solution and incubate for 5 minutes.
3. Pellet the stained aggregates once more and resuspend in a small volume of distilled water. Refer to **Note 12**.
4. Place the stained fibril suspension on a slide and cover with distilled water for imaging.

3.3.2.4 *Imaging of birefringence*

1. Examine the sample on a light microscope.
2. When the polarisers are aligned, Congophilic fibrils will appear pink to red. When the polarisers are crossed at a 90° angle to one another incident light will be blocked and the background will become dark. Under these conditions amyloid fibrils bound to Congo Red will appear as bright green areas in the stained sample. This birefringence is due to the ability of the bound dye to alter the

polarization of the incident light and allow its transmission through the objective.

(See **Notes 14 and 15**)

3.4 Measuring thioflavin T reactivity

Thioflavin T (ThT) is another diagnostic amyloid dye originally developed as a histological stain (17,18). It was subsequently to be used *in vitro* to measure amyloid-like fibrils (19). In essence, the assay detects large spectral changes in the fluorescence properties of ThT when bound to amyloid fibrils, which can be measured with a fluorimeter. Hence, the assay works best in a comparative manner between the non-aggregated protein versus the aggregated counterpart.

Simple spectroscopic assay (See **Note 16**)

1. Dilute stock ThT to 10 μM in approximately 10 ml phosphate buffered saline.
2. Immediately measure the fluorescence intensity of 1 ml of this solution in a fluorescence cuvette using excitation at 445 nm (5 nm bandwidth) and emission at 482 nm (10 nm bandwidth), and average over several seconds.
3. Prepare a solution of the non-aggregated protein in a final concentration of 10 μM ThT. Generally 10 – 100 $\mu\text{g/ml}$ of protein is sufficient.
4. Measure the fluorescence intensity of this solution using parameters as above.

This measurement serves as a negative control to reference against the (expected) increased fluorescence yield observed with the aggregated protein (in step 5).

This control is important to account for ThT binding of the unaggregated form of the protein.

5. Prepare a solution of aggregated protein in a final concentration of 10 μM ThT using the same protein concentration as that in step 3, and measure fluorescence intensity as before. If the protein is amyloid, there should be a significant, and commonly very large, increase in fluorescence intensity relative to the unaggregated form of the protein. (See **Note 17**)

3.5 Kinetics of aggregation

Once it has been established that the protein has aggregated, it is often useful to determine the kinetics of aggregation and/or how environmental parameters might affect the kinetics. Doing so first requires an understanding of the oligomeric state of the native form of the protein in native-like buffers (eg, is it monomeric, dimeric or some other assembly state in phosphate buffered saline), and preparation of the protein to ensure that the native state is free of large amyloid aggregates, which will be the baseline, time zero reference sample.

There is no one-size fits all method to determine these properties of a protein. However a good place to start is to measure the oligomeric size of the native state using gel filtration chromatography in a buffer that matches the native environment of the protein. Other more sophisticated and high resolution techniques for assessing oligomeric state include analytical ultracentrifugation and dynamic light scattering (these techniques will not be covered in this chapter). Some proteins, particularly small proteins and peptides, can be stored denatured at high concentrations, such as in 5 M guanidine hydrochloride, and freshly refolded into the native state by directly diluting out of the denaturant. However, the efficiency of refolding and potential aggregation also needs to be assessed when using this approach.

Another important parameter is whether the native state can be stored frozen in aliquots, which enables more flexibility and convenience in performing the kinetic assays. A general framework for preparing the native protein for kinetic assays is provided here, which will require optimization using the guidelines described above for each particular protein. The volumes shown in the procedure here may need to be scaled depending on protein quantity required for downstream analysis, and type of kinetic assay to be performed.

1. Take sample of the native form of the protein, and remove all pre-existing aggregates by either high-speed centrifugation at 100,000 *g* as described above in section 3.1 step 6, or by gel filtration chromatography as describe above in section section 3.2.
2. Immediately place native protein on ice and set aside a small aliquot to measure the protein concentration.
3. Before measuring protein concentration, aliquot protein into single use volumes in microcentrifuge tubes and snap freeze in either liquid nitrogen, or alternatively, a dry ice-ethanol bath. See **Note 18**.
4. Place the aliquots at -80 °C (for storage up to 6 months.) See **Note 19**.
5. Using the protein set-aside, measure the concentration of the protein using a standard assay or by measuring the absorbance at 280 nm and molar extinction coefficient. Refer to **Note 3**. It is important to measure the protein concentration precisely for quantitative kinetic assays, since protein concentration strongly influences the aggregation rate of most amyloid proteins.

6. Thaw an aliquot of the protein and test whether the protein remains in the native oligomeric state by gel filtration chromatography as described in section 3.2 or by other high resolution techniques such as analytical ultracentrifugation.
7. If the protein has formed aggregates by the freeze-thawing process, then either omit the freezing step for storage (note that the kinetics can still be performed, however with less flexibility). Alternatively, optimize the buffer conditions to avoid the formation of aggregates by freeze-thawing.

Once the protein preparation is complete, it is possible to perform the amyloid-fibrillization kinetic assays using one of the three following methods, the *reverse kinetic assay*, the *forward kinetic assay* and the *continuous ThT assay*. See **Note 20**.

3.5.1 *Method 1: Reverse kinetic assay.*

8. Devise the time points and time frame of the assay. Keep in mind that the assay will be run in reverse with the longest incubation times started first, and with aliquots progressively taken out of storage for decreasing incubation timepoints. At the time of the last aliquot (time point 0), all samples will be measured simultaneously for aggregation. Typically, fibrillization occurs on a time scale of minutes to days, however this can vary widely depending on the protein and environmental conditions. A good starting point for 0.3 mg/ml protein is a 72 h time course, with time points heavily weighted over the first 24 h.
9. Take an aliquot of protein out of -80 °C storage (or dilute out of denaturant).
10. Place in an incubator, PCR machine, or heat block, to regulate temperature.
11. Note down the time the tube was thawed.

12. Once the tube has warmed (several seconds to a minute), open tube and pipette mineral oil (~200 μ l) to cover surface area of sample. This step is crucial to prevent buffer evaporation and condensation on the lid of the tube. However, the mineral oil can be omitted if using a PCR machine equipped with a heated lid option that prevents such condensation.
13. Close lid and continue incubating.
14. Repeat steps 8-12 for each decreasing time point.
15. For the last tube, thaw briefly at the incubation temperature and note the time of thawing.
16. Measure amyloid formation using the assays described (sections 3.1-3.3) above for each aliquot. To remove the sample from the tube, carefully insert pipette tip below the mineral oil and aspirate slowly. It should be straightforward to pipette without drawing oil. See **Note 21**.

3.5.2 Method 2: Forward kinetic assay

17. This assay uses a continuous protein solution, or parallel array of tubes, which will all begin the assay simultaneously and measured at time points of incrementing time.
18. Thaw sufficient aliquots for assay (one aliquot per time point) and pool together. Alternatively, if the protein cannot be freeze-thawed, take freshly prepared protein as described above in steps 1-6.
19. Place protein in an incubator at a regulated temperature.
20. Overlay the protein with mineral oil as described above in step 12.

21. At the first timepoint, remove an aliquot from the tube and assay for amyloid as described in step 18.
22. Repeat step 21 for each timepoint until assay is complete.

3.5.3 Method 3: Continuous ThT kinetic assay

23. Prepare ThT stock and fluorimeter as described in section 2.3. This protocol is most suited to use with a multi-well plate and plate reader (the following protocol will assume this setup). See **note 16**
24. Set plate reader fluorimeter to read fluorescence of the plate at defined intervals over a period appropriate for the time course of fibril formation. See **Note 22**.
25. Prepare protein for fibril formation in the presence of 10 μ M ThT.
26. Place aliquots of this preparation in plate wells and seal with plate-sealing film.
27. Immediately, place plate in plate reader and begin data collection.

4. Notes

1. A recommended high speed ultracentrifuge setup is the Beckman Optima TLX ultracentrifuge and Beckman Coulter TL120.2 rotor with clear polycarbonate tubes (cat# 343778 from Beckman Coulter). The clear tubes facilitates visibility of the pipette tip relative to the supernatant and protein pellet when pipetting.
2. It should be noted that buffer compositions (eg salt concentrations, pH) can strongly influence the formation and rates of amyloid fibrils, their stability/morphology and conversely the stability of the native state of the protein.
3. The protein can be detected directly by the extinction coefficient at 280 nm, however this is not recommended for samples potentially containing aggregates,

- which can have significant turbidity contributions at 280 nm. Hence, protein concentrations should be measured using standard protein detection assays such as Lowry (20), bicinchoninic acid (21), or Coomassie Blue based approaches (eg Bradford assay (22)). Such assays also provide more flexibility in protein volumes and concentrations and can be adapted to a plate reader format for large sample handling. Inexpensive kits are available from most bioscience vendors.
4. To readily identify the pellet, mark the outside face of the tube with a dot. It is important that the supernatant be removed by slow aspiration away from the pellet to avoid disturbing the pelleted protein.
 5. If using gravity flow to elute the proteins, ensure that the column outlet contains a valve to so that the flow can be regulated. Alternatively the Econo-pac columns from BioRad are designed to stop flowing when the reservoir above the column has emptied, which is particularly useful for gravity flow applications. If using a peristaltic pump, place a flow adaptor to the top of the column to ensure that the sample is applied evenly to the resin.
 6. Typically the buffer should contain at least 150 mM salt to minimize non-specific binding of the protein to the resin (eg 20 mM Tris, pH 7.8, 150 mM NaCl). Note also that long, thin columns provide the best resolution for gel filtration.
 7. If using a peristaltic pump, adjust the flow adaptor to remove any gap that may have formed from further resin compaction and settling.
 8. When packing and running columns be careful not to run the flow-rate faster than the manufacturer's recommendation because the resin can compact over time

- leading to blocked flow – often in the middle of a crucial run! Also, be sure to run the column with at least 1.5-2 column volumes for each run to ensure material binding non-specifically is fully eluted.
9. Blue dextran 2000 (GE Healthcare) works well for labelling the void volume on a sepharose CL-2B column.
 10. In the first instance it is worth trying 2 μ M Congo Red with as high a protein concentration as possible, but the ratio and final concentrations may need optimizing depending on the protein.
 11. Other proteins that are not in an amyloid-state may also produce a similar result and hence it is important to also measure birefringence, which is a more conclusive indicator of amyloid fibrils.
 12. At this stage it is possible to wash the stained fibrils by repeated pelleting and resuspension in distilled water, if required to remove excess stain.
 13. This method is good for concentrating protein aggregates from large or dilute volumes.
 14. The thickness of the sample can have a large effect on the observation of birefringence. Thus, a number of different samples of different spread and thickness should be examined.
 15. Many things are capable of producing birefringence under cross polarized light (e.g. hair and other natural fibers, dust, salt crystals). For this reason, false positives and misinterpretation can occur. Detection of strong *green* birefringence corresponding to areas stained pink/red under bright-field examination is generally considered positive for amyloid. Ideally, a known amyloid sample, such

- as A β should be examined in parallel. See Fig 2 shows the birefringence for a protein prepared by Option C.
16. The ThT fluorescence assay can be easily adapted for multi-well plates and using a fluorescence plate reader. This allows the assay volume to be substantially reduced, thereby reducing consumption of protein. To do this, modify the assay so that the final concentration of ThT is 10 μ M. Hence in a 96-well format, pipette together 200 μ l 12.5 μ M ThT in PBS with 50 μ l protein solution.
 17. The fluorescence yield of ThT can be quite different in the presence of different types of amyloid fibrils. Thus, it may be necessary to vary the protein concentration used in the assay to optimise signal and gain statistically significant results.
 18. If using dry ice ethanol bath be careful not to submerge cap of microcentrifuge tube under the ethanol which can lead to ethanol leaking into the tubes. Also use an alcohol-resistant marker on the tubes to avoid labelling washing off.
 19. Do not store at -20 $^{\circ}$ C because at this temperature the ice becomes crystalline rather than vitreous which commonly facilitates protein aggregation. For this same reason, it is important to use the snap-freeze procedure to avoid crystalline ice formation caused by slow freezing.
 20. The *reverse kinetic assay* can only be performed if the protein can be freeze-thawed as described above without inducing aggregation or if the protein can be diluted directly out of a denatured stock without causing spontaneous aggregation. The forward kinetic assay has the advantage of working with proteins that cannot be freeze-thawed. However, drawbacks of this assay are that there is the potential

for baseline drifts over long periods (especially for measuring ThT reactivity) and that it is more time consuming. The continuous ThT assay offers the huge advantage in that it is simple, and easy to scale up, however has a major potential drawback in that the ThT can change fibrillization rates and hence this effect needs to be considered when using this assay.

21. A small amount of oil carryover from the outside of the tip usually will not interfere with the assays for amyloid formation. The most convenient assay for kinetic analysis is the ThT assay in plate reader format. A multichannel pipettor is very handy for large scale kinetic analysis of the ThT assay in the plate reader format.
22. It is important that the fibril suspension is adequately mixed before each fluorescence measurement, as fibrils can become flocculent and sediment quickly to the bottom of the plate well. If your plate reader has a 'mix' functionality, program the instrument to thoroughly mix the plate before each data point collection.

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Figure Legends

Figure 1 – Gel filtration chromatography to separate apoC-II monomers from fibrils as described in section 3.2. The protein (0.5 ml) was loaded onto a 1 x 30 cm column packed in Sepharose CL-2B. The column was eluted in 100 mM sodium phosphate, pH 7.4 by gravity flow.

Figure 2 – Red-green Congo Red birefringence of amyloid fibrils formed by apolipoprotein C-II. The protein aggregates were prepared according to Option C in Section 3.3.2.3. The upper panel is the brightfield view of the Congo-Red stained aggregates. The lower panel shows the green birefringence under cross-polarized light.

Figure 3 – Fibril formation time course for apolipoprotein C-II (open circles) and a peptide fragment derivative of apolipoprotein C-II, apoC-II₅₆₋₇₆ (filled circles) monitored by the *Method 2: Forward kinetic assay* ThT assay (section 3.5.2). The ThT fluorescence intensity has been standardized to the maximum ThT fluorescence intensity signal. However, the ThT fluorescence yield in the presence of apoC-II₅₆₋₇₆ fibrils is approximately 5 fold higher than that in the presence of full length apoC-II fibrils.

Fig 1.

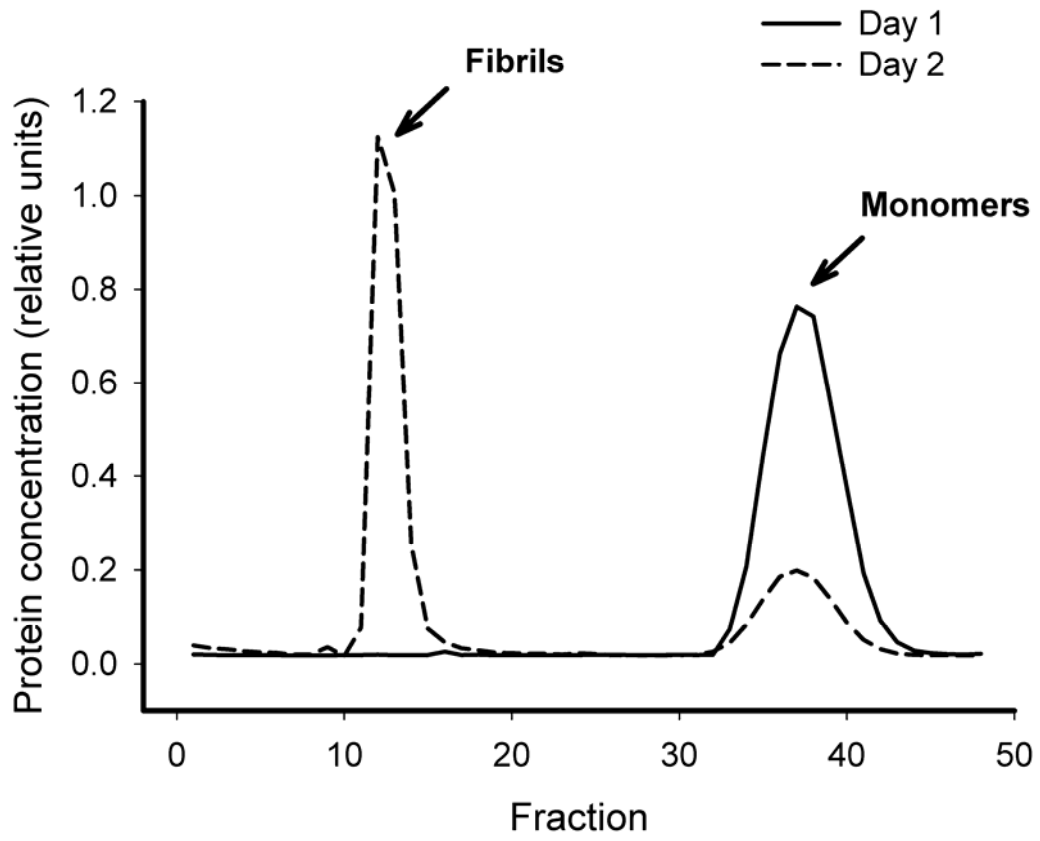


Fig 2.

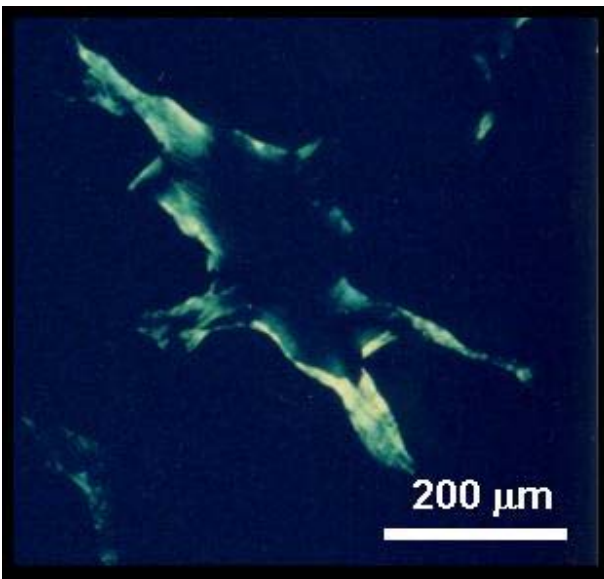
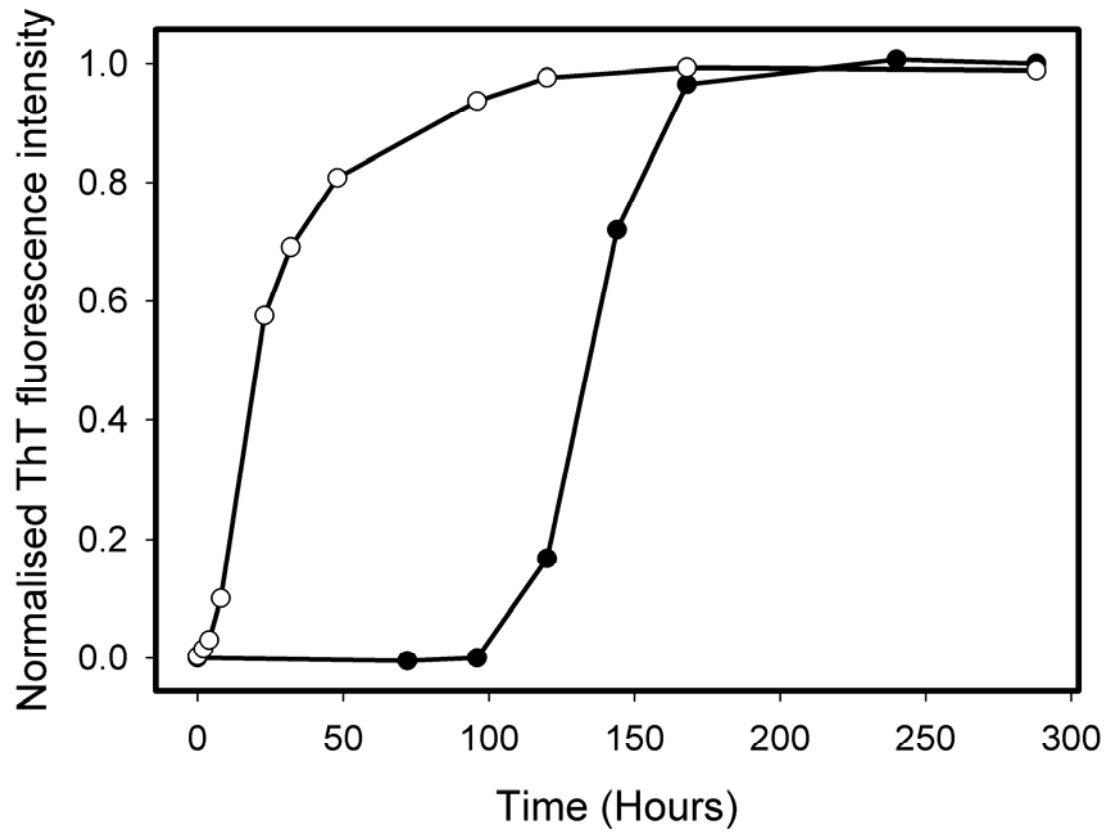


Fig 3.





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