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Assays for Light Chain Amyloidosis Formation and Cytotoxicity

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Abstract

Common biophysical techniques like absorption and fluorescence spectroscopy, microscopy, and light scattering studies have been in use to investigate fibril assembly for a long time. However, there is sometimes a lack of consensus from the findings of an individual technique when compared in parallel with the other techniques. In this chapter, we aim to provide a concise compilation of techniques that can effectively be used to obtain a comprehensive representation of the structural, aggregation, and toxicity determinants in immunoglobulin light chain amyloidosis. We start by giving a brief introduction on amyloid assembly and the advantages of using simple and readily available techniques to study aggregation. After an overview on preparation of protein to set up parallel experiments, we provide a systematic description of the in vitro techniques used to study aggregation in AL protein. Additionally, we thoroughly discuss the steps needed in our experience during the individual experiments for better reproducibility and data analysis.

Keywords

Immunoglobulin light chain; Amyloidosis; Amyloid formation; Thioflavin T; Electron microscopy; Sedimentation assay; Cell viability; Caspase assay

1 Introduction

Amyloid disease initially starts from soluble proteins that polymerize through multiple stages into insoluble amyloid fibrils which get deposited in various organs, leading to organ dysfunction and tissue degeneration [1-3]. The aggregation pathway involves multiple species that self-interact in a variety of ways to produce insoluble amyloid fibrils.

Simple and readily available techniques like thioflavin T (ThT) fluorescence assay and electron microscopy in conjunction with complementary techniques as high performance liquid chromatography (HPLC) sedimentation assay, dynamic light scattering (DLS), and size exclusion chromatography (SEC) can provide a global insight of the whole aggregation process. Experiments with these techniques are easy to set up, accurate and due to minimal technical variability are highly reproducible. Composite analysis of the data obtained from these complementary techniques at periodic intervals of time can provide substantial quantitative information concerning transformation of different species into amyloid assembly. We will focus on the experience we have had with immunoglobulin light chain (AL) amyloidosis. In the following section, we describe our laboratory techniques developed to study in vitro aggregation of AL proteins (Fig. 1) and assays to analyze cytotoxicity and apoptosis in AL amyloidosis. The various subsections in the chapter include preparation of AL proteins and general setup for aggregation kinetic experiments, use of thioflavin T (ThT)

to study fibril formation in vitro, electron microscopy to study the morphology of fibrils and intermediate species, use of HPLC-based sedimentation assay for quantitative determination of soluble proteins present in aggregate suspension, particle size analysis by dynamic light scattering (DLS) to explore the involvement of low molecular weight oligomers (LMW) in the aggregation pathway, and size-exclusion chromatography (SEC) analysis of the soluble aggregate suspension to characterize the gradual transformation of soluble proteins and LMW oligomers into protofibrillar and fibrillar forms, nanoparticle tracking analysis (NTA) to detect species ranging from 30 to 1000 nm in size, and in vitro assays to analyze cytotoxicity and apoptosis induced by fibrils/soluble proteins in light chain amyloidosis.

2 Materials

2.1 Preparation of AL Variable Domain Proteins for In Vitro Assays

Use ultrapure water (prepared by purifying deionized water, to attain a conductivity of 18 MΩ-cm at 25 °C) and analytical grade reagents for all solutions. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials. We do not add sodium azide to reagents.

1. Frozen aliquots of the protein of interest.
2. Bucket of ice.
3. Vortex.
4. 0.22 µm membrane syringe filters.
5. 10 mL conical tubes.
6. 10 mL conical centrifugal filter concentrators.
7. 5 mL polyallomer centrifuge tubes, or equivalent.
8. Ultracentrifuge.
9. UV-vis spectrophotometer.

2.2 Thioflavin T (ThT) to Study Amyloid Fibril Formation In Vitro

1. 10 mM ThT stock solution: 32 mg ThT to 10 mL of ultrapure water and filter through a 0.2 µm syringe filter (*see Note 1*). Determine the real ThT concentration after filtration by UV absorbance spectroscopy using a molar extinction coefficient of 26,620 M⁻¹ cm⁻¹ at 416 nm [4, 5]. Store the solution protected from light (*see Note 2*).
2. 10 mM ABC buffer pH 2–11: 10 mM of sodium acetate, boric acid, and sodium citrate buffer at the desired pH. 0.68 g of sodium acetate, 0.32 g of boric acid, and 1.48 g of sodium citrate, in 500 mL of ultrapure water. Filter the buffer solution using a 0.22 µm filter and aliquot into 10 mL conical tubes. Measure the pH of each conical tube and adjust the pH with either 1 M HCl or 1 M NaOH accordingly (*see Note 3*).

3. 5.0 M NaCl: 14.61 g of NaCl in 50 mL of ultrapure water. Filter it using a 0.22 μ m filter. Store it at room temperature.

2.3 Kinetic Data Analysis

1. Microsoft Excel and/or any scientific graphing and data analysis software, i.e., Origin software package (Origin lab, USA), GraphPad Prism (GraphPad Software Inc., USA).

2.4 Negative Staining of Amyloid Fibrils for Transmission Electron Microscopy (TEM)

1. 0.4% uranyl acetate: 400 mg of uranyl acetate in 10 mL of filtered ultrapure water.
2. Formvar/carbon 300 mesh copper TEM grids.
3. Filter paper.
4. TEM grid tweezers.

2.5 Sedimentation Assay by HPLC

1. HPLC grade water.
2. HPLC grade acetonitrile.
3. HPLC grade formic acid.
4. C8 RP- HPLC analytic column.
5. HPLC chromatographic system.

2.6 Particle Size Analysis by Dynamic Light Scattering (DLS)

1. Cuvette-based DLS molecular sizing instrument.
2. DLS-cuvette (12 μ L square quartz microcuvette).
3. Long round gel loading pipette tips.
4. Ultrapure filtered water.

2.7 Particle Size Analysis by Size Exclusion Chromatography (SEC)

1. Analytical chromatographic system.
2. Analytical size exclusion column with a broad separation range, we recommend agarose-dextran based matrix (we used Superdex 200 size exclusion column with a separation range of 10,000–600,000 Da).
3. Molecular weight protein markers.
4. Phosphate buffer at pH 6.8 as the mobile phase for the size exclusion experiments: 7.09 g of sodium phosphate dibasic, 5.99 g of sodium phosphate monobasic, and 8.76 g of sodium chloride. Adjust the pH of the solution using 1 M NaOH. Bring the volume to 1 L using ultrapure water, mix well and filter through a 0.22 μ m membrane filter.

2.8 Nanoparticle Tracking Analysis

1. 1 mL disposable Luer lock syringes.
2. Phosphate buffered saline (PBS), pH 7.4. (filtered, free of all particulates).
3. Ultrapure H₂O.
4. 10% ethanol for cleaning.
5. Clean tubes and pipets for making sample dilutions.
6. External hard drive disk for large files.
7. Lens paper.
8. Can of compressed air.
9. NanoSight NS300 with software package NTA 2.3.5.

2.9 In Vitro Assays for Light Chain Amyloidosis Cytotoxicity

This protocol is ideal for AC16 human cardiomyocytes cells and mesenchymal stromal cells (MSCs) (*see Note 4*).

1. Cell Culture Media—DMEM:F12 (1:1) (Dulbecco's modified Eagle medium and Ham's F-12 medium), L-glutamine, 15 mM HEPES, 12.5% fetal bovine serum (FBS), 1% penicillin–streptomycin (P/S). Remove and discard 67.5 mL of media from bottle, add 62.5 mL of FBS and 5 mL of P/S to DMEM/F12 media bottle. Filter using a 500 mL, 0.22 µm vacuum driven sterile filter bottle. Store at 4 °C.
2. 0.25% trypsin–EDTA.
3. Fetal bovine serum (FBS).
4. Sterile phosphate buffered saline (PBS), pH 7.4.
5. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) reagent: 10 mg of MTT reagent in 5 mL of sterile PBS buffer, pH 7.4. Filter solution using a sterile syringe filter with a 0.22 µm pore size hydrophilic polyvinylidene difluoride (PVDF) membrane. Store at 4 °C, protected from light (*see Note 5*).
6. Dimethyl sulfoxide (DMSO).
7. Trypan blue stain 0.04% solution.
8. Sterile syringe filter with a 0.22 µm pore size hydrophilic PVDF membrane.
9. CO₂ incubator.
10. Sterile pipette tips.
11. Centrifuge with rotor for 96-well plates.
12. T75 cell flasks.
13. Flat-bottom sterile 96-well polystyrene plates (cell culture quality).
14. Visible/UV plate reader.

15. LDH cytotoxicity detection kit (according to manufacturer's instructions, Clontech).
16. Homogeneous Caspases Assay, fluorimetric kit (according to manufacturer's instructions, Roche).

2.10 In Vitro Assays to Analyze/Study Apoptosis in Light Chain Amyloidosis

1. Cells—chosen and treated based on the experimental model (*see Note 6*).
2. Complete cell culture medium of choice (varies by cell type).
3. Phosphate buffered saline (PBS), pH 7.4.
4. Methanol–acetic acid (3:1) solution (MeOH–AcOH).
5. 50% glycerol–50% 0.1 M Tris–HCl (pH 7.4) containing 1 µg/mL Hoechst 33258 (*see Note 7*).
6. Glass microscope slides and coverslips.
7. Fluorescence microscope possessing the appropriate excitation and emissions filters (*see Note 8*).

3 Methods

3.1 Preparation of AL Variable Domain Proteins for In Vitro Assays

After purification and confirmation of the biophysical properties of your protein or peptide of interest [6–10], it is important to take into account the storage conditions of the protein and peptide prior to the amyloid formation reaction. We particularly suggest aliquoting 1 mL of the proteins at ~100 µM, in 1.5 mL cryogenic tubes, flash-freezing in liquid nitrogen, and storing at –80 °C. In the case of synthetic peptides, it is a common practice to store them as lyophilized peptides on a desiccator, and/or under anaerobic conditions to prevent oxidation of the peptides. These recommendations work well with the light chains proteins in our laboratory, but other proteins may vary in their optimal storage conditions.

1. Thaw frozen aliquots accordingly to the total amount of protein estimated for the experiment, on ice (*see Note 9*).
2. Once thawed, gently mix the content of the tubes by a gentle vortexing. Pool the protein from all the tubes in 5 mL polyallomer centrifuge tubes and spin at in an ultracentrifuge at $645,000 \times g$ (*see Note 10*) (Fig. 1; A). This step removes oligomers, protofibrils and large aggregates (if any) formed during the storage process.
3. Gently remove the top three-fourth part of the supernatant and filter through 0.2 µM low protein binding membrane filter. Additionally you can check the filtered protein solution by SEC and dynamic light scattering (described in Subheadings 2.6 and 2.7) for aggregates, and to obtain a reference point for the soluble protein before the assays (Figs. 1C1 and 2).

4. Mix the filtered protein solution with the buffer of election at desired pH (*see Note 11*) and concentrate (we recommend 350–400 μ M, but it has to be tested for each protein) using conical centrifugal filters with the appropriated molecular weight cutoff (MWCO) (*see Note 12*). This concentrated protein (stock protein) will be used to get the desired concentration for all the experiments reported below (Fig. 1C2).
5. Determine the concentration of the solution by UV absorbance.
6. Prepare the aggregation reaction mixture in 1.5 mL microcentrifuge tube by addition of the 150 mM NaCl [11] and diluting it with an appropriate volume of buffer to the desired concentrations and volume.
7. This reaction mixture would subsequently act as a parallel starting point for ThT binding assay, electron microscopy, HPLC sedimentation assay, DLS, and SEC (Fig. 1; D1) (*see Note 13*).
8. Always keep the protein stock on ice during the entire preparation process.

3.2 Use of Thioflavin T (ThT) to Study Amyloid Fibril Formation In Vitro

Thioflavin T (ThT), a benzothiazole dye (Fig. 3) [12–14] has been widely used for the staining of amyloid fibrils, and is considered the gold standard to quantify and determine the presence of amyloid fibrils in both histological samples, and in vitro assays since its first use as a fluorescent amyloid reporter by Vassar and Culling in 1959 [15–17].

ThT binds equally well to amyloid fibrils and cross β -sheet-rich structures of biological (proteins) and synthetic origin (peptides) [17, 18], displaying a characteristic red shift in the emission spectrum [15, 17, 19], and an enhanced fluorescence [20] when bonded, which makes it a cheap, fast, and reliable amyloid probe [15, 21, 22].

The in vitro spectroscopic assay is commonly used to monitor fibril formation over time, but the assay is not strictly quantitative and some deviations/interferences can be observed. Hence, to rule out interferences in the in vitro assay due particular requirements of the experimental setup, some considerations have to be taken into account, like ensuring the quality and conformational homogeneity of the protein sample prior to the experiment, a correct selection of buffer systems compatible with the ThT dye, protein stability at the desired temperature for the experiment, and the correct preparation of the stock solutions, in particular the ThT solution. In our experience, the ThT assay to monitor kinetics of fibril formation in proteins may be set in two different ways depending on the purpose of the planned experiments:

- a. Low volume ThT assays: 260 μ L of the desired concentration of soluble protein, buffer, NaCl, and ThT are incubated in a black 96-well polystyrene plate (Greiner 96 well black 6550) in an incubator shaker (*see Note 14*).
- b. High volume ThT assays: 1 mL of the desired concentration of soluble protein, buffer, NaCl, and ThT are incubated in low protein binding microcentrifuge tube in an incubator shaker. 300 μ L of the reaction mixture is read on a 96-well microplate thereafter (*see Note 15*).

To study the aggregation pattern of soluble proteins, we generally prepare an aggregation mixture with the desired concentration of soluble protein, selected buffer of election, 150 mM NaCl at the desired pH (see **Note 16**). Typically the aggregation mixture is incubated at 37 °C in low protein binding microcentrifuge tubes with constant shaking at 300 rpm in a temperature controlled incubator with orbital shaking (see **Note 17**). Depending on the number of assays and number of time points to be studied, the volume of the aggregation mixture is kept constant for soluble proteins across individual experiments (see **Note 18**). At the end of the aggregation experiment (when most of the soluble proteins have transformed into fibrillar form) (see **Note 19**), the microcentrifuge tube with the aggregate suspension is frozen at -20 °C. The thawed, washed aggregate suspension is used to study fibril cytotoxicity and apoptosis in light chain amyloidosis cellular model systems.

For the amyloid fibril formation assay in presence of ThT, the protein of interest is incubated with ThT and other relevant buffer components and the ThT signal from each sample is read, often in a plate reader, which allows multiple samples to be measured at once as a function of time [10, 23, 24].

1. Prepare the ThT (see **Notes 1 and 2**), 10 mM ABC buffer (see **Note 3**), and the protein solution stocks at high concentration to allow you pipetting small volumes with confidence (if it is possible, we suggest ~400 µM protein stock), to reach the final concentration desired (see **Note 20**). Keep the protein stock on ice during the entire preparation process.
2. Add the required volume of ABC buffer to a microcentrifuge tube.
3. Add the required volume of 5.0 M NaCl stock to get a final concentration of 150 mM.
4. Add the required volume of protein stock to get a final concentration of 20 µM.
5. Add 10 µM equivalent of ThT to each tube, and mix well by vortexing (keep it on ice until this time) (see **Note 21**).
6. Check the pH of the sample and adjust if necessary (see **Note 22**).
7. Aliquot the samples in black 96-well polystyrene plate (Greiner 96-well black 6550), seal the wells with sealing film, cover the plate with a black lid, and seal it with tape, to decrease evaporation (see **Note 23**).
8. Record how much time it takes you from the time you start adding protein until you take the initial reading in fluorescence plate reader for time corrections.
9. Take an initial reading of the fluorescence emission at time zero, on a plate reader using an excitation wavelength of 440 nm, and 480 nm for the emission (see **Note 24**).
10. Shake plate at 300 rpm, 37 °C in a temperature-controlled incubator. We prefer orbital shaking over linear shaking.
11. Take subsequent readings at regular intervals. In cases that the kinetic is really slow, take daily readings at specific time for up to 1 month (~750 h). For fast

kinetics, we recommend use a plate reader in kinetic mode. For other reading settings in your reader, please refer to the manual of your equipment, and adjust the settings accordingly.

3.3 Kinetic Data Analysis

Analyzing in vitro amyloid fibril formation kinetics is not trivial; misleading conclusions can be drawn due to incorrect analysis. A thorough discussion of the models to analyze fibril formation kinetics is beyond the scope of this chapter (for a good review on the topic, see [25, 26]). We will present an empirical approach to analyze and extract kinetic parameters from experimental data, as well as the use of an empirical sigmoidal function (logistic function) to fit kinetic data. The logistic function is not directed to any specific molecular process, but it is a good entry point to study the molecular mechanism of amyloid aggregation.

1. Prepare the kinetic data by plotting the ThT fluorescence values as a function of time. It is preferable to analyze the kinetic traces of each replicate as independent data because averaging fibrillation curves can introduce bias (see **Note 25**). Export the ThT fluorescence data as a function of time into a spreadsheet. We advise you to use the first row to name the columns with descriptive headers, especially if your data consist of many experiments.
2. We do not recommend normalizing the kinetic traces (see **Note 26**).
3. Verify that all the data is in the same units, as well and check for any outlier data. ThT fluorescence data could be noisy.
4. Next, choose/delimit the region that which will be to fit to obtain the lag, elongation phase, and plateau value. To determine the lag and plateau phases there should be enough data points for the fitting, but not to include any signal before the plateau is reached.
5. A classic example of a ThT-monitored kinetic curve is shown in Fig. 4. Such a curve may be analyzed using the sigmoidal logistic function [23]:

$$Y = y_i + m_i t + \frac{y_f + m_f t}{1 + e^{-[(t - t_{50}) / \tau]}} \quad (1)$$

where Y is the fluorescence intensity as a function of time t , y_i and y_f are the intercept of the initial and final baselines with the y -axis, m_i and m_f are slopes of the initial and final baselines, t_{50} is the time needed to reach halfway through the elongation phase and τ is the elongation time constant. The apparent rate constant, k_{app} , for the growth of fibrils is given by $1/\tau$, and the lag time is usually defined as $t_{lag} = t_{50} - 2\tau$.

Or using the most general sigmoidal function, the Boltzmann equation (defined as a Boltzmann function by the Origin software package <http://www.originlab.com/www/helponline/Origin/en/UserGuide/Boltzmann.html>).

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} + A_2 \quad (2)$$

Where A_1 is the initial fluorescence value, A_2 is the final fluorescence value, x_0 is the center ($t_{1/2}$ value), dx is defined as the time constant. In both cases, a larger t_{50} value indicates a longer time required to form fibrils (see **Note 27**).

6. Once all the data has been preprocessed, data fitting can be performed using your favorite scientific graphics and data analysis software (see **Note 28**).
7. In the case of noisy kinetics data, with deviations from the sigmoidal shape, it is a common procedure to determine the t_{lag} and/or t_{50} of your reaction by fitting the elongation phase using a linear function. The t_{lag} is the intercept between linear extrapolations of the lag phase (Fig. 4, dashed lines) and the elongation phase respectively (Fig. 4, continuous lines) (see **Note 29**).
8. The ThT endpoint intensity could be proportional to the amount of amyloid formed. This correlation differs among proteins and peptides; therefore it has to be determined/standardized for each protein by an independent assay, such as measuring the remaining soluble protein at the end of the reaction.

3.4 Negative Staining of Amyloid Fibrils for TEM

Amyloid fibrils are insoluble particles in the nanometer-micrometer-scale in length. This wide size range makes TEM an excellent method to visualize and validate the presence of amyloid structures [27], gain knowledge of the morphological properties of the aggregates and discriminate between protofibrils, mature fibrils and amorphous aggregates [28].

The most common TEM techniques is the negative staining with 2% uranyl acetate [29], using 200–400 mesh spacing copper that are carbon coated and covered in formvar.

1. Spin down the 4% uranyl acetate solution at $13,000 \times g$ for 3 min to pellet any insolubilized uranyl acetate.
2. Filter the uranyl solution using a 0.22 μM . This solution can be stored for several weeks at 4 °C. Be aware that uranyl acetate may contain some traces of radioactive uranium and therefore the solution and the powder should be stored appropriately.
3. Place 3 μL of protein sample on the grid. After 3 min, apply a torn edge of hardened, ashless filter paper at the edge of the grid to absorb the excess liquid. (This process is known as wicking.)
4. Immediately place 3 μL of 2% uranyl acetate solution on the grid, wait 1 min, wick away excess solution, and air-dry (see **Note 30**).
5. The grids can be immediately examined or stored in an EM grid case prior to examination.

6. The grids can be imaged using an electron microscope operating at 80 keV (*see Note 31*).

3.5 Sedimentation Assay by HPLC

Sedimentation assay followed by HPLC for tracing amyloid transformation was developed by the Wetzel's laboratory [30] to study aggregation in A β and polyglutamine peptides. HPLC sedimentation assay is a quick and ingenious way to quantitate the concentration of the unreacted monomer in the aggregate suspension. In this section we describe how to use this method effectively to study the aggregation of globular proteins with minor modification of the original method described by the Wetzel laboratory.

The HPLC sedimentation assay consist of two parts: (1) creation of a standard curve; which requires plotting the integrated area obtained from HPLC peak against the mass of the soluble AL protein in the sample and (2) determining the concentration of the soluble protein present in the aggregation reaction mixture from the integrated peaks during the course of aggregation. It is important prior to running the experimental samples, to standardize the instrument and to characterize the reverse phase HPLC separation properties of your protein (including the choice of reverse phase column, flow rate, injection volume, gradient of the solvents, and wavelength of the detector). Once the set of parameters are standardized, the method works well for most of the protein/peptides studied. To study AL proteins, we used C8 (2.5 $\times$ 50 mm) analytical reverse phase column and a gradient of acetonitrile in 0.1% TFA maintained at 300 μ L/min flow rate. The wavelength of the UV detector is fixed at 280 nm to selectively absorb the aromatic residues in proteins.

1. Prepare the AL proteins as described in Subheading 3.1 (Fig. 1A and B1). Make serial dilutions of the protein with the highest possible accuracy, using the protein stock and reestimate the concentration of the serially diluted samples by UV absorbance (Fig. 1C1). These samples are used to plot the standard curve (Fig. 5).
2. Inject the different samples into an RP-HPLC column (being consistent with the volume injected every time) in triplicates to ensure the quality of the calibration curve.
3. Construct the standard curve by plotting the integrated peak areas (obtained from reverse phase HPLC) as a function of protein concentration (calculated from UV-absorbance spectroscopy) and fit the curve to a liner regression. The slope of the curve obtained represents area/ μ g (Fig. 5).
4. The averaged value of the slope obtained from the triplicates is used to determine the concentration of unreacted soluble protein during the course of aggregation reaction (*see Note 32*).
5. After preparing the samples for aggregation reaction as described in Subheading 3.1, inject immediately $\sim$ 20 μ L aliquot (in triplicates) in the RP-HPLC column and note the concentration for the zero time (total soluble protein at the beginning of the reaction) (Fig. 1D1).

6. After injecting protein samples in the HPLC, place microcentrifuge tubes in the orbital shaker at 37 °C and 300 rpm and/or quiescent conditions as per the requirements of the aggregation experiment (*see Note 33*).
7. At predetermined time intervals, transfer an aliquot (typically 80 µL) of the suspension into an ultracentrifuge tube, spin at $160,000 \times g$ for 45 min in an ultracentrifuge at 4 °C (*see Note 34*). Repeat the process till the concentration of soluble protein plateaus at minima.
8. Carefully transfer 40 µL of the centrifuged solution and mix it with 20 µL of 1% formic acid and inject it in the reverse phase HPLC (*see Note 35*).
9. After every sample injection, blank injection (with the similar gradient as sample injection) was interspersed to ensure proper flushing of any residual protein from the column before the next run.
10. To determine the aggregation kinetics, plot the protein concentration (calculated from the integrated area and the standard curve for individual protein sample) as a function of the time elapsed.

3.6 Particle Size Analysis by DLS During Aggregation Reactions

Analysis of particle size by dynamic light scattering (DLS) is a versatile and accurate way of determining the monodisperse or polydisperse distribution of species during protein assembly. DLS computes the autocorrelation function by measuring the intensity of light scattered by the particles undergoing Brownian motion. Fitting of the autocorrelation function thus obtained provides diffusion coefficients of particles which can then be used to calculate the hydrodynamic radius from Stokes-Einstein equation [31].

DLS can be accurately employed to study the early events during the course of aggregation process. It suffers, however, from major drawbacks while studying the entire aggregation spectrum as during the elongation phase scattering of light might predominantly be contributed from a fraction of amyloid assembly in the suspension. Fibril length, flexibility and self-interaction in the amyloid assembly during the elongation phase might also lead to complexity in the mutual diffusion [31]. Although these complexities can be expressed by multiexponential correlation function and the apparent hydrodynamic radius calculated, the data is not reliable and leads to overestimation of particle size in a polydisperse sample.

1. For this protocol, we are using the cuvette-based DLS with 1 µM upper size limit for detection of hydrodynamic radius, as reference.
2. Prepare the samples of aggregation reaction for measurement as described in Subheading 3.1.
3. Turn on the DLS instrument, and start the software.
4. In the software interface of the instrument, set the viscosity and refractive index of the solvent for water before the start of scattering measurement. Also select the power for the laser beam.

5. Immediately take an aliquot of ~30 μ L, and gently pipette it into cuvette using a gel tip (*see Note 36*).
6. Insert the cuvette into the DLS instrument cell holder and tightly close the lid to start the measurement. Repeat the process at predetermined time intervals.
7. Check the average intensity autocorrelation function, from at least 30 independent acquisitions from samples at early time point (*see Note 37*).
8. As the light scattered from a solution is extremely sensitive to sample impurities and dust, after every sample measurement the cuvette is cleaned several times in soap solution and ultrapure water.
9. Dry any excess of water sticking to the cuvette by compressed air.

3.7 Size Exclusion Chromatography (SEC)

SEC is a popular and wide spread analytical tool to study the in vitro amyloid aggregation, given its ability for quick interpretation of data and separation based on size/molecular weight (MW) of various species coexisting at any given time. Further, the variations resulting from shape and equilibrium condition among the heterogeneous population of aggregates is largely minimized due to significant difference in molecular weight between aggregates and prefibrillar species. The chromatogram obtained has optimal resolution and can be used to demonstrate a concomitant appearance or disappearance of species over a period of time. Furthermore the species detected during the course of aggregation can easily be isolated and processed for structural analysis and toxicity assays.

1. Prior to sample injection, filter all the solvents used as mobile phase (phosphate buffer at pH 6.8) using 0.2 μ M membrane filters (*see Note 38*).
2. Inject molecular weight protein markers to obtain a standard curve for the column, by plotting the elution volume against MW of the protein standards (*see Note 39*).
3. Inject approximately ~150 μ L of the aggregation reaction mixture (preparation described in Subheading 3.1).
4. Determine the apparent molecular weight from the elution volume of individual chromatographic peaks using the standard curve.
5. After a few injections, intercalate blank samples to verify the baseline of the column.

3.8 Nanoparticle Tracking Analysis

Similar to DLS, Nanoparticle Tracking Analysis (NTA) utilizes light scattering intensity from particles undergoing Brownian motion to determine the hydrodynamic radius of individual particles [32]. Due to the low refractive index of protein, particles ranging from 30 nm to 1 μ m are ideal for NTA analysis [33]. NTA differs from DLS in that particles flow and are tracked using video and tracking software to simultaneously determine the values for a multitude of individual particles per frame. The characteristics/properties of the sample population are thus available and overcome the problem of large aggregates within

polydisperse samples obscuring the majority sample signal in DLS. With NTA, it is also possible to visualize sample particles in real time and results are available quickly. This is an advantage over electron microscopy and other methods prone to preparation artifact [34]. Calculations assume a spherical shape, so irregularly shaped particles are difficult to measure reliably. Thus, it is ideal to complement NTA analyses with methods such as DLS and electron microscopy to confirm measurements.

1. Assemble sample chamber, optical flat, and attach tubing. Insert chamber into the Nanosight NS300 device and power it on.
2. Launch NTA 2.3.5 Software Stage and Temperature Comms.
3. Launch software interface and within the main menu, **Connect Syringe Pump**.
4. Select **Capture** to activate camera display (*see Note 40*).
5. Load 1 mL disposable Luer lock syringe with ddH₂O. Expel a small amount of liquid against the sample syringe port to ensure there are no bubbles and attach. Slowly advance the plunger to advance approximately 900 µL of liquid through the chamber (*see Note 41*).
6. Watch the camera display to ensure there are no air bubbles and the chamber is filling with fluid. Once the chamber is filled with fluid, adjust the **Camera Level** to the highest level that does not produce excessive background.
7. Repeat syringe flush at least twice to ensure that there are no contaminating particles left in the lines from the last user or air bubbles (*see Note 42*).
8. Load one syringe filled with PBS dilution buffer into the chamber, and check that it is indeed particle free.
9. Prepare diluted samples of particles of interest in PBS buffer. You will need at least 1 mL volumes to fill the sample syringe for measurement (*see Note 43*).
10. Fill a clean 1 mL syringe with sample, attach to syringe port, and now load syringe into the syringe pump. Run at Infusion rate of '250' until sample particles begin to appear on the screen. Slow the infusion rate down to an infusion rate of '25' (*see Note 44*).
11. Adjust **Focus** and **Detection Threshold** until you can clearly see particles with small light halos around them. Record these settings and keep them consistent for samples you want to compare between.
12. Select **Capture Duration** based on polydispersity of samples. For 10–40 particles per frame and medium polydispersity, the recommended capture is 60 s. For highly polydisperse samples, 90 s is preferable.
13. Click **Record**. Complete sample description screens and make folders for the data to be exported to. Be sure to record the dilution factor used (1:1000, etc.), the camera level, and detection threshold. Save when prompted.
14. **Process Sequence** to analyze the video and obtain data. Excel and PDF files will be exported and include a graphical representation of particle size vs.

concentration, a numerical list of particles of particular bin sizes with concentrations, and finally a sample population mean, mode, standard deviation, and overall sample concentration. For disperse samples, the mode tends to be more descriptive than the mean values.

15. Multiple videos may be recorded from the same syringe, but consider sample settling in the syringe when evaluating values. Sometimes running the infusion pump rate at '250' for several seconds will sufficiently agitate the sample that you can get another good measurement (*see Note 45*).
16. Flush the lines with sufficient ddH₂O to ensure no particles are seen between syringes of sample.
17. Finally, the chamber must be cleaned with ddH₂O, 10% ethanol, and air. Chamber must be disassembled and dried thoroughly with compressed air and lens paper before storage (*see Note 46*).

3.9 In Vitro Assays for Light Chain Amyloidosis Cytotoxicity

MTT and LDH Cytotoxicity Assays are colorimetric assays to quantitatively measure cellular cytotoxicity (*see Note 47*). MTT assay quantifies cell proliferation by measuring the mitochondrial activity of the succinate dehydrogenase enzyme. LDH assay measures lactate dehydrogenase enzyme (LDH) released into the extracellular culture media from damaged cells. Both methods result in a formazan product that can be measured spectrophotometrically.

Always warm the culture media to 37 °C in incubator or water bath, 15 min before use. The age of the cultures, number of passages, and details of the growth medium are important factors for accurate cell viability results. Be consistent between experiments and avoid cells reaching confluent growth and high passages (< passage #25).

1. Thaw the cells by gently warming the vial tube containing the cells for 2 min at room temperature and 2 min at 37 °C.
2. Transfer contents of vial (approximately 1 mL) to 50 mL conical tube containing 24 mL of prewarmed media and mix gently.
3. Transfer all contents into a T75 cell culture flask.
4. Place flask in incubator overnight.
5. Next day, check for cell growth and change media.
6. Cell passage is kept at 20 before disposal.
7. To count the cells, aspirate cell media with a glass pipette.
8. Wash the cells with 25 mL PBS solution twice.
9. Add 3 mL 0.25% trypsin–EDTA to cells.
10. Put flask in incubator for no more than 2 min.
11. Gently rock the flask to help cell detachment.

12. Add 10 mL PBS to flask to inhibit the trypsin effect.
13. Transfer all contents of the cell flask into 50 mL conical tube.
14. Centrifuge the 50 mL conical tube at $225 \times g$ for 5 min, at 22 °C.
15. Resuspend the pellet with 2–4 mL of prewarmed media until solution looks homogeneous.
16. Transfer 100 μ L of cell/media solution to a 1.5 mL microcentrifuge tube and vortex.
17. Gently mix 10 μ L cells with 10 μ L of trypan blue stain 0.04% and apply 10 μ L into a Countess Cell Chamber slide.
18. Count viable cell concentration using a Cell Countess.
19. Calculate the volume of cell/media solution that you need for splitting 3×10^5 cells in a new T75 flask. (3×10^5 AC16 cells usually take 3 days to reach overconfluence.)
20. Transfer the cells to a 50-mL conical tube containing 25 mL of prewarmed media and mix gently.
21. Transfer all contents into a T75 cell culture flask.
22. Place flask in incubator overnight.
23. Next day, check for cell growth and change media.
24. Calculate the volume of cell/media solution that you need for preparing 25 mL of prewarmed media containing 10,000 cells per mL.
25. Transfer the cells to 50-mL conical tube containing media and mix gently.
26. Plate 200 μ L of cells at a concentration of 10,000 cells per mL (2000 cells/well) in a 96-well polystyrene plate.
27. Incubate the cells at 37 °C, 5% of CO₂, and allow them to reach 70% of confluence (within 24–48 h) before addition of the soluble protein.
28. To prepare the amyloid fibrils for the cell culture, if frozen, thaw the amyloid fibrils at room temperature from your stock, and gently vortex to resuspend the fibrils (see Note 48).
29. Dilute the samples to approximately 2 μ M (substock) with cell culture media, such that the final concentration of fibrils in 96-well plate is around 1 μ M.
30. Resuspend 68 μ L from the substock in 1.6 mL of AC16 stock media.
31. For the co-culture experiments for cell growth effects, seed AC16 cardiomyocytes in a 96-well plate (see previous protocol).
32. Place the 96-well plate in the IncuCyte Incubator (see IncuCyte Protocol) and allow the cells to adhere to the bottom of the plate for 24 h.
33. Remove 96-well plate from incubator and remove media.

34. Add 100 μ L of fresh DMEM:F12 and 100 μ L of 2 μ M fibril substock solution to each well of the plate. This concentration is a 1:1 preparation; dilutions may be created to achieve the desired concentration of plated cells, fibrils, and other components such as soluble proteins (*see Note 49*).
35. Place 96-well plate back in incubator. Plates can be scanned every 4 h or as needed.
36. Follow the incubation for 6 days using IncuCyte. IncuCyte ZOOM software was used to count cells by measuring number of cells with red fluorescence protein (RFP).
37. For the caspase activity assay, seed the cells as described above with either 1 μ M fibrils or 1/12 μ M soluble protein. Cell viability can/must be measured for each experimental condition (fibril/soluble protein) by 80 h using the IncuCyte ZOOM (*see Note 50*).
38. After 80 h, 100 μ L of substrate working solution was added to each well of a 96-well plate. Incubate the plates for 1 h, at 37 $^{\circ}$ C, 5% CO₂.
39. Following incubation, read the plate using a fluorescence plate reader with the following setup: 480 nm on the excitation wavelength and 520 nm for the emission wavelength, using medium attenuator mode with a continuous lamp.
40. The collected data must be reported as averaged fluorescence units/well, from triplicates of each well and each condition.
41. For the assay using soluble protein, remove the media by aspiration and replace it with 100 μ L of fresh media (*see Note 47 for LDH assays, see Note 51 for MTT assays*).
42. Add 100 μ L of soluble protein to the cells at a different final protein concentration (we recommend concentrations within the range of 1–20 μ M). Dilute the soluble proteins to the appropriated concentration using fresh cell culture media.
43. Incubate cells with soluble protein for various time points (24, 48, and 72 h).
44. Include a negative control made of 200 μ L media alone (blank), along a positive control of cells without proteins (control).
45. For the mitochondrial viability assay, replace the cell media with 100 μ L of fresh media without norepinephrine and phenol red (*see Note 51*).
46. Add 10 μ L of 5 mg/mL MTT stock solution to each well (final concentration of 0.5 mg/mL per well) (*see MTT reagent preparation in Subheading 2.9*), and incubate the plate at 37 $^{\circ}$ C, 5% of CO₂, for 4 h.
47. Add 100 μ L of DMSO to each well and incubate them overnight to solubilize the precipitates.
48. Read the absorbance in a plate reader device at 570 and 690 nm.

49. Subtract the 690 nm absorbance reading (reference) from the 570 nm absorbance values for each well.
50. Calculate the percentage of living cells using the following formula:

$$\% \text{ Living cells} = (\text{sample absorbance} - \text{blank absorbance}) \times 100 / (\text{control absorbance} + \text{blank absorbance}).$$

51. For the Lactose Dehydrogenase Assay, transfer the 200 μL of media from each well into a new 96-well polystyrene plate.
52. Centrifuge the plate at $300 \times g$ for 10 min, at 22 $^{\circ}\text{C}$.
53. Transfer 100 μL of supernatant to a new plate carefully, without disturbing the cell pellet.
54. Add 100 μL of freshly prepared reaction mixture to each well, and incubate it at room temperature for 30 min, protected from light.
55. Read the absorbance in a plate reader device at 492 and 690 nm.
56. Subtract the 690 nm absorbance reading (reference) from the 492 nm values for each well.
57. Calculate the cytotoxic effect using the following formula:

$$\% \text{ Cytotoxicity} = (\text{sample absorbance} - \text{spontaneous absorbance}) \times 100 / (\text{maximum absorbance} - \text{spontaneous absorbance}).$$

3.10 In Vitro Assays to Analyze/Study Apoptosis in Light Chain Amyloidosis

Multiple studies implicate AL amyloid light chains in the induction of apoptotic events in a variety of target cells [35-40]. Consequently, it can be advantageous to utilize methodologies that detect characteristic apoptotic changes [41, 42] resulting from amyloidogenic cytotoxicity. Many indicators of apoptosis can be quantified via biochemical analyses. Common examples include: Caspase assays, which detect the activation of cysteine aspartate-specific proteases [43]; dUTP nick end labeling (TUNEL) assay to detect DNA fragmentation [44]; and Annexin V assay for flow cytometric detection of translocated cell-surface phosphatidylserine [45]. Alternatively, analyses of characteristic morphologic changes to apoptotic cells can be utilized. Among these, DNA staining of viable cells with fluorescent bisbenzimidazole (Hoechst) dyes in conjunction with fluorescence microscopy allows for ready visualization of chromatin condensation and related apoptotic events [46, 47].

Hoechst dyes offer some of the most frequently used reagents for dsDNA staining and characterization. The Hoechst dyes, in particular Hoechst 33258, permeable to intact cell membranes, bind to the minor groove of DNA at regions rich in AT sequences, and fluorescence bright blue emission upon excitation by ultraviolet light when in the bound state. This fluorescence intensifies with the staining of condensed chromatin, characteristic of apoptotic cells, allowing for an observable distinction from non-apoptotic cells [48].

Additional indicators of apoptosis, such as nuclear shrinkage and apoptotic body formation, can also be visualized with this method.

1. For staining DNA with Hoechst 33258, transfer an aliquot of treated cells from a suspension or prepared adherent culture to a 15 mL conical tube (*see Note 52*).
2. Sediment the cells by centrifugation at $225 \times g$ for 10 min.
3. Aspirate the supernatant medium, reserving a nominal amount ($\sim 100 \mu\text{L}$) of fluid above the cell pellet. Resuspend the cells by gently flicking the tube.
4. Wash the cells by adding 10 mL PBS and sediment by centrifugation at $225 \times g$ for 10 min.
5. Aspirate the supernatant PBS, again reserving $\sim 100 \mu\text{L}$ of fluid. Gently resuspend the cell pellet by flicking the tube (*see Note 53*).
6. Fix the cells by slowly adding $200 \mu\text{L}$ 3:1 MeOH:AcOH and gently mix with the cell suspension.
7. Add an additional 2 mL 3:1 MeOH:AcOH solution to the cells, mix gently, and incubate for 1 h at room temperature.
8. Sediment the cells by centrifugation at $225 \times g$ for 10 min.
9. Aspirate the MeOH:AcOH, leaving $\sim 400 \mu\text{L}$ in the tube.
10. Resuspend the fixed cells in the remaining liquid.
11. Deposit fixed cells onto a glass microscope slide by dispensing $100 \mu\text{L}$ of the cell suspension dropwise using a micropipette with a fine tip.
12. Allow the slide to air-dry.
13. Dispense a drop of 50% glycerol–50% 0.1 M Tris–HCl (pH 7.4) containing $1 \mu\text{g/mL}$ Hoechst 33258 to cover the cells.
14. Overlay a glass coverslip onto the slide. Affix the coverslip by sealing the edges with a thin layer of clear nail polish. Allow the sealant to air-dry (*see Note 54*).
15. Observe the samples using a fluorescence microscope with ultraviolet excitation.

4 Notes

1. The solubility of ThT on ethanol (100 g/L) is higher than on water (5 g/L), however water is preferred.
2. A stock solution ($\sim 10 \text{ mM}$ ThT) protected from light and stored at 4°C could last for several weeks without apparent damage, as long as they are carefully protected from light [49]. The concentration range between 10 and $60 \mu\text{M}$ ThT provides a good working concentration [23, 50].
3. The 10 mM sodium acetate, boric acid, and sodium citrate buffer (ABC) presents buffering capacity through a broad range of pH values (from pH 2 to 11). ABC buffer stored at room temperature last for up to 1 year.

4. The cell line has been immortalized by fusion with SV40 transformed fibroblast cell line devoid of mitochondrial DNA.
5. MTT stock can be stored at 4 °C for several months. It is recommendable to filter it before using it.
6. Suspension and/or adherent cell lines should be maintained using standard but cell-specific laboratory practices. Protocols for utilizing AC16 cardiomyocytes, which were analyzed in this staining method, are detailed in the “*In Vitro Assays for Light Chain Amyloidosis Cytotoxicity*” section of this chapter.
7. There are three related primary Hoechst dyes: Hoechst 34580, Hoechst 33258, and Hoechst 33342, the latter two being the most commonly used for DNA staining [48]. Hoechst staining solutions can be stored at 4 °C for several weeks when protected from light [51].
8. Hoechst 33258 and Hoechst 33342 exhibit similar excitation/emission spectra (–350 nm/460 nm) [52]. This protocol utilized a microscope equipped with an excitation filter of 365 nm and a long pass emission filter of 420 nm.
9. It is important to estimate the amount of protein needed, prior to the experiment, in order to reduce variability associated with the use of different lots of protein. We recommend using an excess of protein in approximately the volume needed for three samples to avoid being short on protein.
10. We determine the correct speed ($645,000 \times g$) needed to sediment preformed aggregates, using the following equation:

$$\log s = \log k + \frac{2}{3} \log M$$

$$t = \frac{k}{s}$$

where s = sedimentation coefficient.

k = Clearing factor (specific for the rotor used).

M = Molecular wt (of the species to be removed)

11. Mixing the centrifuged solution with your selected buffer at the desired pH before concentration is recommended as this step works as a buffer exchange, which eliminates the need to readjust the pH while setting up aggregation experiment.
12. It is desirable to use a concentrated stock of protein whenever it is possible (–400 μ M). However this depends on the solubility and stability of the protein under investigation. We suggest performing an experiment testing the dependence of aggregation as a function of the protein concentration.
13. We recommend taking aliquots for all the assays from the same reaction mixture at periodic intervals of time. The rationale behind this is to have a complete set of data that can be extrapolated over each other, if required, to accurately

determine any variations in the structure and assembly of AL proteins during the course of aggregation.

14. The ThT assay on black 96-well polystyrene plate is mainly done to explore the changes in kinetics of fibril formation in soluble protein and ensure reproducibility with reported results. The reactions in microcentrifuge tubes display different kinetics, possibly due to the differences in the surface composition and the different air–water interfaces.
15. In order to monitor the stepwise conversion of soluble protein to mature fibrils and correlate it to kinetics of fibril formation, we prepare two sets of aggregation mixture, with and without ThT in low protein binding microcentrifuge tubes. Since subtle changes in the vessel geometry and material of the container affects the kinetics of fibril formation, we recommend using identical low protein binding microcentrifuge tubes to prepare the aggregation mixture with and without ThT. Aggregation mixture without ThT can be used to observe structural changes in the soluble protein and formation of intermediate species during the aggregation pathway which can later be used to correlate with the kinetics of fibril formation as determined by ThT assay.
16. We typically use 10 mM ABC buffer to study aggregation in AL proteins as it has a broad pH range (pH 2–11) which provides an opportunity to monitor AL proteins aggregation kinetics at different pH values. Most of the AL proteins aggregate at pH 2.0 and 4.0.
17. Care should be taken to set the incubator shaker at 37 °C at least an hour prior to the start of the experiment.
18. Maintaining a constant volume of aggregation mixture across individual experiments minimizes error in reproducibility.
19. The end of the aggregation reaction is precisely determined by either by ThT assay (described Subheading 3.2) or HPLC sedimentation assay (described in Subheading 3.5).
20. This allows for flexibility with amyloid formation assays as a function of protein concentration. In case you are using a different buffer, adjust the buffer concentration accordingly to keep the desired buffer concentration unaltered.
21. The use of a master mix prepared in the same way for every experiment is the best way to achieve consistency and good initial ThT values.
22. Reactions that occur at pH 8 or higher pH values and/or elevated temperatures can result in hydroxylation of ThT, resulting in photo bleaching [53, 54].
23. You can add water/buffer to all the unused wells, as well as seal the lids with lab tape to reduce the evaporation.
24. The initial readings may be unusually high, especially at some pH values; this is related to ThT reequilibration at the experimental temperature—Take a new measurement after 5 min and discard the high value from the analysis.

25. In general, fibril formation reaction data should be acquired until the reaction is completed (i.e., until a clear plateau value is reached). However, it is possible that the plateau region will present some fluorescence decay possibly due to photo bleaching.
26. Analyzing the kinetics of each replicate independently helps to avoid misinterpretation of the results, e.g., biphasic traces or poor reproducibility or the strong stochastic amyloidogenic character of some proteins.
27. When preparing the samples, the protein should be kept on ice until the beginning of the measurement, and the measurement is usually taken above room temperature (e.g., at 37 °C). Thus, because there will be thermal reequilibration of the sample as the experiment is being recorded and incubated (usually not more than 5 min) the signal may vary unexpectedly—for example, it may decrease because of the dependence of fluorescence emission with temperatures, and make sure not to include these initial data points into the analysis.
28. Microsoft Excel software provides a lot of flexibility to processing data; however nonlinear fitting and statistical analysis can be easier using more specialized software.
29. The concentration of ThT positive species in the lag phase is far below the detection limit, and therefore there will be only baseline ThT signal before a critical concentration of ThT positive species are formed [2]. The t_{50} value describes the time required for the ThT fluorescence intensity to reach the half point of the reaction; along with an apparent elongation rate (slope of the transition) that is associated to the rate of recruitment of soluble precursors to the growing fibrils. These parameters allow us to characterize the amyloid formation reactions in terms of the lag time and the elongation rate. The plateau at the final phase indicates the end of the reaction.
30. Careful handling of the grids is required to avoid artifacts that can be mistaken for protein aggregates. Some imperfections on the formvar by a poor coating can appear to be interesting shaped protein aggregates. In addition, a high amount of protein or aggregates can result in a “black grid” (over staining). However with practice and experience these artifacts can be easily detected and fixed.
31. Scanning the grids at low magnification (10,000–12,000×) can give an idea of the overall sample composition and then proceed to examine the grids at higher magnification (25,000×). Amyloid fibrils are typically linear, unbranched, and 5–10 nm in width; however it is not uncommon to find big tangles of fibril. Also, it is not uncommon to observe different aggregate morphologies on a single grid [9, 24], coexisting at any given time point. TEM is complimentary to the ThT assay.
32. The use of triplicates potentially minimizes the pipetting errors and errors associated with the HPLC autoinjection.
33. Depending on your needs, you can perform the fibril formation assay under quiescent or shaking conditions. We suggest performing a pilot experiment to

determine which condition is the best for your specific protein/system. In general shaking of samples results in a more efficient amyloid fibril formation compared to quiescent conditions, however the amyloid fibrils obtained under quiescent conditions are longer and less tangled.

34. Most of the amyloid like aggregates and protofibrils can be pelleted at $160,000 \times g$. Elimination of preformed aggregates can be critical on the determination of the kinetics of amyloid formation, especially if you have interest in the kinetic parameters of the reaction.
35. Formic acid is known to prevent aggregation in some peptides in the HPLC vials. We have found that in case of AL protein, in the presence of 1% formic acid in the HPLC vials, aggregation is negligible for up to 6 h as determined by dynamic light scattering.
36. Using a gel tip to dispense sample significantly reduces introduction of air bubble inside the cuvette.
37. The hydrodynamic radii and percentage mass (density measure of the population density) of individual particles in the aggregation reaction mixture is measured from the average intensity autocorrelation function computed by the software provided with the instrument.
38. The filtration of the mobile phase is mandatory, especially if the solvent remain unused for at least a week. Avoid filtering the samples before injection as it could eliminate some low molecular weight oligomers that could be interesting to characterize.
39. The election of the correct SEC column is critical for the determination of the apparent molecular weight of the species present/populated during early events of the fibril formation reaction.
40. Camera sensitivity (camera gain and shutter speed) and software settings will dramatically affect the ability to detect particles. This is defined within the NTA software package as Camera Level CL. To distinguish particles from background, it is possible to specify a Detection Threshold (a minimal light intensity obtained from a particle to identify it as a particle) and a Minimum Track Distance (the area of light must move against background to qualify as a particle).
41. High pressure will damage the system, so always introduce liquid slowly into the port.
42. An air bubble can be detected by the sample particles running at a deflected angle across the screen rather than horizontally. To remedy this, slowly flush out a syringe filled with air into the sample port, and then reflush with fluid until sample runs horizontally again.
43. Optimal concentrations will provide 10–40 particles per frame, which are approximately 10^7 – 10^9 particles per mL. This is critical to obtain clear images of individual particles without overlap. The Capture Time/Duration (long capture

times result in extremely large data files) and whether the sample is monodisperse vs polydisperse will influence the ideal dilution factor and may require multiple attempts to optimize. If you discover that the sample you have introduced is excessively concentrated, stop advancing and immediately flush with copious amount of ddH₂O. Highly concentrated samples can dirty the lens and contribute to background.

44. For good reproducibility between experiments, it is critical to be consistent with the settings for image capture and run comparable samples together whenever possible. Chamber components are sensitive to damage by caustic solutions and solution at extreme pH values. The need to neutralize samples prior to NTA measurement may adversely affect labile protein aggregates and require use of an alternate method.
45. For our studies, triplicate samples are typically taken from each syringe with 10 s “pushes” in between.
46. Day to day changes in chamber age/cleanliness/background can dramatically affect measurements by requiring adjustments to Camera Level and Detection Threshold. The chamber component sensitivity is the primary reason why chamber cleanliness/background is a factor. This makes the chamber difficult to clean well, so they are meant to be disposable when excessively dirty. However, they are not inexpensive, so it is ideal to get as many measurements from them as possible.
47. Both human and animal sera contain LDH, which may increase the background absorbance of the assay. Therefore, for LDH assays, replace the 12.5% FBS media for 1% FBS fresh media before adding proteins to the cells. Follow instructions from LDH Cytotoxicity Detection Kit User Manual.
48. For fibril formation procedures see reference [40].
49. Fibrillar substocks must be vortexed frequently to ensure a good homogeneity when adding to the 96-well plate. Amyloid fibrils are heavy and will collect at the bottom of tube, thereby skewing the total amount of fibrils plated per well.
50. Apoptotic reagent CellPlayer kinetic caspase-3/7 (Essen Bioscience) was initially used as it allowed for kinetic quantification of cell proliferation over time. However, this reagent became bound to the external amyloid aggregates and emitted a green fluorescence to give off a false positive signal. In order to avoid false positives, the caspase assay kit can be used (Roche) following the instructions from Homogeneous Caspases Assay, fluorimetric kit User Manual.
51. The presence of phenol red in the cell culture media may affect the results of the mitochondrial viability assay. Therefore, for MTT assays, it is recommended to replace the phenol red-containing media for phenol-red free media before adding proteins to the cells.
52. The treatment groups for these experiments included AC16 cardiomyocytes incubated with 12 μ M soluble amyloidogenic light chains. Additionally, control

groups for comparison included negative (media only), apoptosis positive (camptothecin induced), as well as controls for any specific solvents in use (e.g., DMSO).

53. Depending on cell type, the cell pellet may not always suspend readily with simple flicking of the tube. Should this be the case, a p1000 pipette may be used to gently draw the cells up and down to create a uniform suspension. In any instance, vigorous agitation of the cells should be avoided.
54. Prepared sample slides can be stored in the dark at 4 °C.

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Fig. 1.

Schematic representation of the laboratory techniques we used to study in vitro aggregation of AL proteins. A: After pooling thawed AL protein (from -80°C) in the centrifuge tubes, spin at $643,867 \times g$ for 3 h 20 min. B1: Decant three-fourth parts of the centrifuged solution into 10,000 MWCO centrifugal filter and concentrate to 350–400 μM . B2: Decant three-fourth parts of the centrifuged solution into 10,000 MWCO centrifugal filter mix ABC buffer pH 2.0 and concentrate to 350–400 μM . C1: Serially dilute the concentrated AL protein with Tris buffer pH 7.4 and plot the HPLC standard curve and determine the reference point (in terms of particle size and MW) for individual soluble AL proteins from DLS and SEC. C2: Dilute the concentrated AL protein with ABC buffer pH 2.0 to the desired concentration and add 150 mM NaCl. This is the starting reaction mixture for the aggregation reaction kinetics. D1: As soon as the aggregation reaction mixture is prepared, parallel reading in DLS, SEC, and HPLC is obtained to test the quantity and quality of soluble protein. D2: After taking the first measurements, setup the reaction mixture in an incubator shaker (37 $^{\circ}\text{C}$; 300 rpm). E1: At predetermined time intervals take required aliquots from the reaction mixture in the incubator shaker for DLS and SEC experiments. E2: At predetermined time intervals take aliquot ~80 μL and centrifuge at $160,000 \times g$ for 45 min. F: Mix 40 μL of the supernatant from step E2 with 20 μL of 1% formic acid and inject in the analytical reverse phase HPLC to determine the concentration of unreacted monomers

Fig. 2.

Example of a serial dilution of AL protein (κ I 018/08 V_L) with Tris buffer pH 7.4 to obtain a reference point for soluble protein (see step C1 of Fig. 1). **(a)** Size exclusion chromatogram representing apparent MW observed in κ I 018/08 V_L over a range of concentrations. **(b)** Hydrodynamic radii of particles observed for κ I 018/08 V_L over a range of concentrations. The hydrodynamic radii of the particles are mentioned on top of the graph. Values in parentheses represent mass percentage (population density) of individual particles

Fig. 3.
(a) Chemical structure of Thioflavin T. (b) Fluorescence emission of unbounded ThT (black line) and bounded ThT to fibrils (red line), $\lambda_{\text{excitation}} = 440 \text{ nm}$ and $\lambda_{\text{emission}} = 480 \text{ nm}$

Fig. 4.

Typical kinetic traces of fibril formation obtained by in situ *ThT assay*. In this example, reactions were performed with 20 μM protein, 10 mM ABC, 150 mM NaCl, at pH 2.0 in the presence of 10 μM ThT. Dashed lines represent the line of best fit. Continuous lines represent the linear extrapolations of the elongation phase; the intersect with the lag phase baseline is the t_{lag} . t_{50} values were obtained by nonlinear fitting to the Boltzman function (Eq. 2) of each independent kinetics, and were averaged from three independent traces. In this example, red circles show data of the amyloidogenic AL-09 V_L protein, and blue triangles represent data from the control κI 018/08 V_L protein.

Fig. 5. Example of a standard curve of protein κ I 018/08 V_L obtained after plotting the integrated area of the peak from reverse phase HPLC against mass in μ g of serially diluted (in Tris buffer pH 7.4) protein