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Author	Family Name	**Caballero**
	Particle	
	Given Name	**Ana B.**
	Suffix	
	Division	NanoBIC, Department of Inorganic and Organic Chemistry, Faculty of Chemistry
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
	Division	Institute of Nanoscience and Nanotechnology (IN2UB)
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
Author	Family Name	**Gamez**
	Particle	
	Given Name	**Patrick**
	Suffix	
	Division	NanoBIC, Department of Inorganic and Organic Chemistry, Faculty of Chemistry
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
	Division	Institute of Nanoscience and Nanotechnology (IN2UB)
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
	Organization	Catalan Institution for Research and Advanced Studies, Passeig Lluís Companys 23
	Address	Barcelona, Catalonia, Spain
Author	Family Name	**Sabate**
	Particle	
	Given Name	**Raimon**
	Suffix	
	Division	Institute of Nanoscience and Nanotechnology (IN2UB)
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
	Division	

		Department of Pharmacy and Pharmaceutical Technology and Physical-Chemistry, Faculty of Pharmacy and Food Sciences
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
Corresponding Author	Family Name	**Espargaró**
	Particle	
	Given Name	**Alba**
	Suffix	
	Division	Institute of Nanoscience and Nanotechnology (IN2UB)
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
	Division	Department of Pharmacy and Pharmaceutical Technology and Physical-Chemistry, Faculty of Pharmacy and Food Sciences
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
	Email	aespargaro@ub.edu

Abstract	Amyloid aggregation is linked to a number of human disorders that range from non-neurological illnesses such as type 2 diabetes to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. The formation of insoluble protein aggregates with amyloid conformation inside bacteria, namely, in bacterial inclusion bodies, offers the possibility to use bacteria as simple models to study amyloid aggregation processes and potential effects of both anti-amyloid drugs and/or pro-aggregative compounds. This chapter describes fast, simple, inexpensive, highly reproducible, and tunable in vitro and in cellulo methods that use bacterial inclusion bodies as preliminary screening tools for anti-amyloid drugs.

Keywords (separated by '-')	Bacterial inclusion body - Amyloid aggregation - Drug screening - Conformational disease

Chapter 12

Anti-Amyloid Drug Screening Methods Using Bacterial Inclusion Bodies

Ana B. Caballero, Patrick Gamez, Raimon Sabate, and Alba Espargaró

Abstract

Amyloid aggregation is linked to a number of human disorders that range from non-neurological illnesses such as type 2 diabetes to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. The formation of insoluble protein aggregates with amyloid conformation inside bacteria, namely, in bacterial inclusion bodies, offers the possibility to use bacteria as simple models to study amyloid aggregation processes and potential effects of both anti-amyloid drugs and/or pro-aggregative compounds. This chapter describes fast, simple, inexpensive, highly reproducible, and tunable in vitro and in cellulo methods that use bacterial inclusion bodies as preliminary screening tools for anti-amyloid drugs.

Key words Bacterial inclusion body, Amyloid aggregation, Drug screening, Conformational disease

1 Introduction

Protein misfolding has been the subject of intense research in recent years, mainly because of the link between the formation of insoluble protein deposits in human tissues and the development of more than 36 human debilitating illnesses, the so-called conformational diseases. Conformational diseases range from non-neurological illnesses such as type 2 diabetes to neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, tauopathies, amyotrophic lateral sclerosis, Huntington disease, or prion diseases [1, 2]. The proteins that are present in these aggregates are found mainly in amyloid conformation. Amyloid fibrils display a core region in cross-β structure, in which repetitive arrays of β-sheets run parallel to the fibril axis [1].

Protein aggregation occurs during recombinant expression in prokaryotic systems and gives rise to insoluble protein deposits called inclusion bodies (IBs); the formation of IBs limits the biotechnological application of bacteria for industrial production and purification of proteins [3]. IBs were long considered as very dense

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cytoplasmic or periplasmic particles of around 1 μm in diameter, without regular secondary structure nor activity, and only useful if refolded [3]. However, it was later found that these apparently amorphous protein agglomerates actually are rich in active, amyloid-type structures [4]. Their β-sheet-rich structures are detectable by X-ray diffraction, Fourier transform infrared spectroscopy (FTIR), and circular dichroism (CD). Fluorescence spectroscopy, ultraviolet/visible (UV/Vis) spectroscopy, and birefringence can be used as well after binding to amyloid-tropic dyes like thioflavins S (Th-S) and T (Th-T), or Congo red (CR). Furthermore, these β-sheets are resistant to proteinase K digestion [5, 6]. IBs form fibrils that can be visualized by both scanning (SEM) and transmission (TEM) electron microscopies and by atomic force microscopy (AFM). They have a seeding capacity reminiscent of amyloids, can form homogeneous aggregates without cross-aggregation, and display aggregation propensities strongly affected by mutations [7].

The formation of IBs is a consequence of the overexpression of amyloidogenic proteins in prokaryotic systems [8]; thus, IBs act as isolated reservoirs of amyloid aggregates [5, 6, 9–12]. Reported data suggest that amyloid-prone proteins involved in human diseases also tend to aggregate and accumulate as insoluble IBs when they are produced in bacteria as overexpressed recombinant proteins [11]. In fact, the recombinant expression of unstructured proteins that are known to develop amyloid structures, such as amyloid β (Aβ) [13], α-synuclein (α-syn) [14], or prion protein (PrP), [15] usually results in the formation of intracellular aggregates associated with the formation of IBs [11]. A similar process can be observed with the recombinant expression of globular proteins that possess high aggregation propensity such as transthyretin [11, 16], β2-microglobulin [17], insulin [18], or lysozyme [19]. Consequently, recombinant protein production is a useful tool to obtain amyloid proteins and their mutated derivatives, which allows to study the aggregation process in a significant number of human conformational diseases related to protein misfolding and fibrillation. Such similarity between the aggregation behavior of amyloidogenic proteins in humans and bacteria permits to use bacteria as a simple model to assess in vivo the activity of potential anti-amyloid drugs. Bacteria represent a highly tunable model in which all amyloid-prone proteins linked to human conformational diseases can be, a priori, overexpressed, hence providing a great platform for the study of amyloid aggregation under various conditions.

So far, the screening of anti-amyloid drugs has consisted of in vitro and in cellulo assays with different cell lines and in vivo tests using transgenic animals. In vitro assays are fast and tunable but often performed too far from the real (natural) conditions; therefore, such assays are suitable for prescreening purposes,

although the results are generally hardly reproducible in vivo. In addition, the anti-amyloid properties of potential drugs are restricted to specific aggregation conditions used in a given in vitro assay, hence possibly leading to significant differences between different (but a priori related) studies. In cellulo methods are increasingly used in the high-throughput screening of anti-amyloid compounds [20]; however, they do not allow to draw conclusions regarding the nature and/or extent of the drug effect, partly because they do not enable a proper monitoring of amyloid aggregation [21]. In summary, tests based on cellular lines are limited to determine mainly cell survival. Considering that a reduction in toxicity (linked to amyloid aggregation) could be indicative of anti-amyloid properties of a potential drug, these assays give indirect information about the effect of drugs on the amyloid process. Recently, in cellulo methods have emerged to assess amyloid aggregation, such as that developed by Cornejo et al., in which the phosphorylation state of the tau protein is used as a reporter of protein aggregation [22–24].

Regarding in vivo assays using transgenic animals, they are certainly essential in the search for potential anti-amyloid drugs. However, in vivo assays using mammals (usually mice, vole, or rabbits) are time-consuming (from months to years) and therefore highly expensive. For this reason, in vivo assays using transgenic animals are normally limited to a very reduced number of carefully selected compounds. To circumvent these limitations, the use of simpler animal models (non-mammals) such as the zebra fish, drosophila, or nematodes is increasing [25–27]. These alternative models cannot replace the use of mammals, but since they are cheaper and faster, they allow screenings of large libraries of drugs, prior to in vivo tests of selected lead compounds. In this context, bacteria (as simple prokaryotic models) and yeast (as simple eukaryotic models) may become alternative models to set up pre-screening systems to test large libraries of potential anti-amyloid compounds through fast, simple, and inexpensive assays [28].

Since IBs can be considered as reservoirs of overexpressed recombinant proteins, mainly aggregated into an amyloid conformation, they may directly be used to assess the activity of potential anti-amyloid drugs. Currently, IBs can be obtained through a simple, fast, inexpensive, highly reproducible, and tunable method that allows to produce partially purified recombinant proteins, mainly aggregated into an amyloid conformation. *In vitro methods* with IBs can thus be used to directly assess the activity of potential anti-amyloid drugs via two different approaches: (i) determination of the *anti-aggregation capacity* of potential drugs and (ii) evaluation of *disaggregating properties*, checking the capacity of drugs to revert or reduce the presence of preformed amyloids (Fig. 1).

Screening methods	Anti-aggregation capacity	End-point measurements	*In vitro*	(section 3.1.3.)
			In cellulo	(section 3.2.1.)
		Time resolved measurements	*In vitro*	(section 3.1.4.)
			In cellulo	(section 3.2.2.)
	Disaggregation properties	End-point measurements	*In vitro*	(section 3.1.5.)
			In cellulo	(section 3.2.3.)
		Time resolved measurements	*In vitro*	(section 3.1.6.)
			In cellulo	(section 3.2.4.)

Fig. 1 Screening methods using bacterial inclusion bodies

Amyloid aggregation in cells and tissues presents several difficulties such as low protein concentration, slow aggregation rates, and low reproducibility. These aspects limit the screening of large libraries of compounds. In contrast, the formation of amyloid structures in prokaryotic cells (bacteria) displays great reproducibility and allows to generate important concentrations of heterologous protein in amyloid conformation. Thus, such *in cellulo methods* permit to follow the amyloid aggregation in living organisms (in a real biological environment) wherein amyloid proteins can interact and associate with other cellular components. Bacterial cells represent a simple in vivo model of amyloid aggregation (viz. in IBs), which can be used to assess the activity of potential anti-amyloid drugs through two different approaches: (i) determination of the *anti-aggregation capacity* of potential compounds and (ii) evaluation of the *disaggregating properties* of potential drugs (Fig. 1).

In the present chapter, in vitro and in cellulo methods using bacterial IBs are described for the study of amyloid aggregation. Such methodologies can be used to investigate both amyloid aggregation and amyloid disaggregation, via end-point and time-resolved measurements.

2 Materials

All the materials required are divided into two main blocks: the first block includes the material needed for the in vitro methods and the second one is required for the in cellulo methods. All solutions were prepared in deionized water (dH_2O).

2.1 In Vitro Methods

2.1.1 Protein Production

1. Culture media. A rich medium such as Luria–Bertani medium (LB) or microbial growth medium 2×YT can be used to grow bacteria.
   (a) Luria–Bertani medium (LB): In 900 mL of dH_2O, add 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl. Mix to dissolve and adjust the pH to 7.0 with NaOH (*see* **Note 1**). Adjust to 1 L with dH_2O and sterilize by autoclaving.
   (b) 2×YT medium: The protocol is the same as for the LB medium but dissolving 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 900 mL of dH_2O. Adjust to 1 L with dH_2O and sterilize by autoclaving.
   (c) LB and 2×YT agar plates: Dissolve agar in LB or 2×YT medium at a concentration of 1.5% (w/v). Pour 20–25 mL of LB-agar or 2×YT-agar into a Petri dish (enough to cover the bottom of the dish) under sterile conditions. Leave the plates standing at room temperature until congealed. Then store them inverted at 4 °C.
2. Antibiotic, stock solutions. The culture media should be supplemented with the appropriate concentration of the corresponding antibiotic. Most of the stock solutions are prepared at a 1000× concentration, namely, 1 mL of stock solution of antibiotic is added to 1000 mL of medium. Filter sterilize with a 0.22 μm syringe cellulose acetate filter. Store as 1 mL aliquots at −20 °C.
3. 1 M Isopropyl-beta-D-thiogalactopyranoside (IPTG) (*see* **Note 2**): Dissolve the appropriate amount of IPTG in dH_2O. Filter sterilize with a 0.22 μm syringe cellulose acetate filter. Store as 1 mL aliquots at −20 °C.

2.1.2 IBs Purification

1. Lysis buffer: 50 mM Tris–HCl, pH 8, 1 mM EDTA, 150 mM NaCl. To prepare 1 L of this buffer, dissolve 6.06 g of Tris–HCl, 0.37 g of EDTA, and 8.77 g of NaCl in 900 mL of dH_2O. Then, adjust the pH with HCl (*see* **Note 3**) and bring to 1 L with dH_2O.
2. 100 mM phenylmethylsulfonyl fluoride protease inhibitor (PMSF): Dissolve the appropriate amount of PMSF in ethanol (*see* **Note 4**). Dispense into aliquots and store at −20 °C.
3. 10 mg/mL Lysozyme: Prepare the corresponding stock solution of lysozyme in dH_2O. Dispense into aliquots and store at −20 °C.
4. 1 mg/mL DNase I and RNase I: Prepare the corresponding stock solutions in dH_2O. Dispense into aliquots and store at −20 °C.
5. 1 M $MgSO_4$ stock solution (*see* **Note 5**): Dissolve 0.12 g of $MgSO_4$ in 1 mL of dH_2O. Store at 4 °C.

6. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 at pH 7.4. In a glass beaker, dissolve 8 g of NaCl, 200 mg of KCl, 1.44 g of Na_2HPO_4, and 245 mg of KH_2PO_4 in 800 mL dH_2O. Adjust the pH to 7.4 and add dH_2O to obtain 1 L of buffer.
7. Nonidet P-40.
8. Triton X-100.

2.1.3 Dye Staining

Since IBs are mostly utilized for the screening of large libraries of potential anti-amyloid drugs, the use of specific amyloid dyes such as thioflavin-T (Th-T), thioflavin-S (Th-S), and Congo red (CR) appears to be the most convenient procedure to track amyloid aggregation in a fast, inexpensive, and reproducible fashion. Since Th-T shows low accumulation in cells, Th-S is more convenient for the in vivo detection of amyloid deposits, including bacterial IBs.

1. Thioflavin-S and T and CR stock solutions: 1% w/v, 250 μM, and 200 mM, respectively, are prepared by dissolving the respective amounts of the dyes in MilliQ water.
2. Th-T/-S and CR solutions are filtered using polycarbonate membranes with a 0.2 μm pore size to remove aggregates.
3. To determine the concentrations of Th-T and CR, molar absorptivities of 36,000 M^{-1} cm^{-1} at 412 nm and 33,000 M^{-1} cm^{-1} at 490 nm are used, respectively (*see* **Note 6**).

2.1.4 Denaturing Agents

1. 8 M Guanidine hydrochloride (GuHCl) stock solution (*see* **Note 7**): Place 76.42 g of GuHCl in a beaker and add water to reach a volume close to 100 mL. Heat the mixture for a few minutes and stir until complete dissolution. Add water up to 100 mL (*see* **Note 8**).
2. 10 M Urea stock solution: Proceed in the same way as with guanidine hydrochloride stock solution (*see* above). In this case, 60.06 g of urea are dissolved in a final volume of 100 mL.

2.2 In Cellulo Methods

2.2.1 M9 Medium

1. Salts M9 10×: To prepare 100 mL dissolve 6.8 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4Cl in dH_2O. Autoclave for 20 min at 120 °C. Store at 4 °C.
2. 40% Glucose: Dissolve 4 g of glucose in 10 mL of dH2O. Sterilize the solution over a 0.22-μm cellulose acetate filter (*see* **Note 9**). Store at 4 °C.
3. 1 M $MgSO_4$ stock solution: Dissolve 2.46 g of $MgSO_4$ in 10 mL of dH_2O. Autoclave and store at 4 °C.
4. 50 mM $CaCl_2$ stock solution: Dissolve 0.055 g of $CaCl_2$ in 10 mL of dH_2O. Autoclave and store at 4 °C.

2.2.2 M9 Preparation

1. To prepare 10 mL of M9 medium, mix 1 mL of salts M9 10×, 100 µL of glucose 40%, 20 µL of 1 M $MgSO_4$ and 50 mM $CaCl_2$ solutions, and 10 µL of the appropriate antibiotic. Add dH_2O up to 10 mL.

3 Methods

3.1 In Vitro Methods

IBs can be used directly to assess the activity of potential anti-amyloid drugs. However, the use of IBs requires their prior purification (Fig. 2).

3.1.1 Protein Expression

1. Day 1: For an overnight culture, a single colony is selected from an LB agar plate of bacterial cells transformed with plasmid encoding for the desired protein and incubated in 5 mL of LB or 2×YT medium with 5 µL of the appropriate antibiotic at 37 °C under agitation (*see* **Note 10**).
2. Day 2: Use 200 µL of overnight culture to inoculate fresh LB or 2×YT medium (10 mL with 10 µL of 1000× antibiotic). Incubate the culture with agitation at 37 °C until the absorbance at 600 nm (OD_{600nm}) reaches approximately 0.6 (*see* **Note 11**).
3. When the culture has reached an appropriate OD_{600nm}, induce the recombinant expression with 10 µL of IPTG (final concentration of 1 mM). The cells are cultured at 37 °C for further 16 h.

3.1.2 IBs Purification

There is a large number of protocols for the purification of IBs. A standard method based on the use of detergents is explained below [29]. The specified volumes are calculated for a culture volume of 10 mL.

1. The first step is the bacterial overexpression of the recombinant protein (*see* Subheading 3.1.1.), after which the cells are harvested by centrifugation at 12,000 *g* and low temperature (4 °C) for 15 min.
2. Discard the supernatant and resuspend the pellet (containing the bacterial cells) in 200 µL of lysis buffer with 36 µL of PMSF and 7 µL of lysozyme, corresponding to approximate final concentrations of 15 mM and 300 µg/mL, respectively. Incubate at 37 °C for 30 min under gentle stirring.
3. Nonidet P-40 at 1% v/v is then added to the sample and allowed to incubate under mild agitation for 40 min at 4 °C. It is important to maintain a mild agitation to avoid precipitation and a correct mixing of the detergent.
4. Thereafter, 4 µL of DNase I, RNase, and $MgSO_4$ are added, and the sample is incubated at 37 °C for 30 min (*see* **Note 12**). After the incubation, the IBs are separated by centrifugation at 12,000 *g* at low temperature (4 °C) for 15 min.

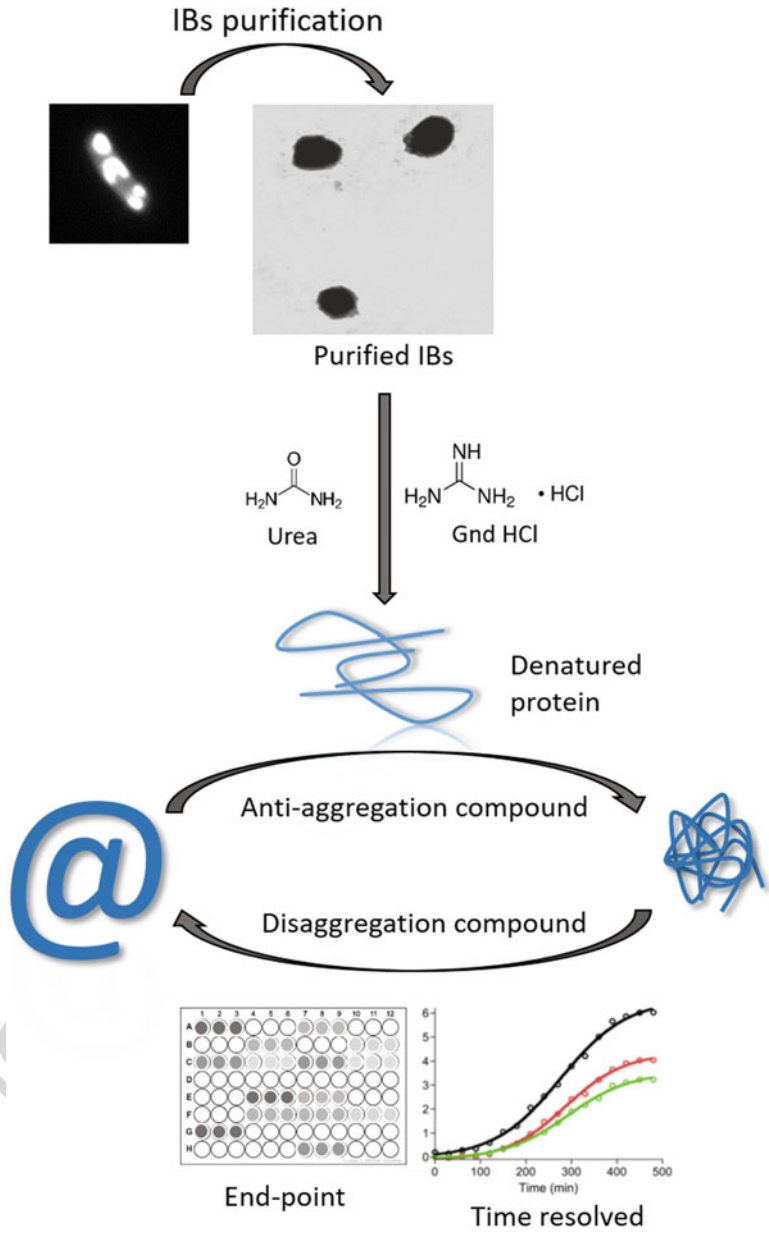

Fig. 2 Outline of the in vitro methods to identify compounds able to modulate protein aggregation. (Modified with permission from [31, 35])

5. Wash the IBs with lysis buffer containing 0.5% Triton X-100. Centrifuge at 12,000 *g* for 15 min, and wash three times with the required buffer, usually PBS or Tris buffer to remove the remaining detergent.
6. After the last centrifugation, the pellets can be stored at −80 °C until analysis [30].

3.1.3 End-Point Amyloid Aggregation or In Vitro Refolding Assay

As previously mentioned, the main advantages of using IBs are the simple preparation of soluble and non-aggregated purified proteins following simple denaturation and refolding protocols and the low price of recombinant proteins and peptides in comparison to synthetic ones.

1. 15 μL of purified IBs at $OD_{360} = 10$ are centrifuged for 10 min at 12,000 *g*.
2. Resuspend the pellet in 10 μL of 8 M GuHCl or 10 M urea and incubate at room temperature for at least 4 h.
3. For the refolding process, denatured aggregates are dissolved in 990 μL of refolding buffer. The refolding buffer contains 880 μL of PBS, 100 μL of Th-T (final concentration of 25 μM), and 10 μL of the compound (to be tested) according to the anti-aggregation assay (*see* **Note 13**).
4. Prepare the appropriate controls. The positive control (maximum fluorescence) is the same buffer without any compound (*see* **Note 14**), the negative control (minimum fluorescence) is that without IBs or compound (*see* **Note 15**), and the compound control is that without Th-T (*see* **Note 16**).
5. The Th-T fluorescence of the samples containing soluble recombinant protein (obtained from IBs denaturation) and of all the controls is measured in triplicate in a 96-well plate with a plate reader or using a fluorimeter, applying excitation and emission wavelengths of 445 nm and 485 nm, respectively.

3.1.4 Time-Resolved Amyloid Aggregation Assay (Amyloid Aggregation Kinetics) (See ***Note 17***)

In vitro amyloid aggregation kinetics can provide complementary information about the aggregation process. An exhaustive kinetic analysis of amyloid aggregation process provides information that can unravel valuable aspects of the mechanism of amyloid aggregation and/or the inhibition effect of a potential anti-amyloid drug. Namely, monitoring the amyloid-aggregation kinetics allows to determine the effect of a given inhibitor at each stage of the aggregation process; this information will shed light on the behavior of each inhibitor along the aggregation process and may allow to identify the main species preferably interacting with the inhibitor.

For the kinetic experiments, the refolding step was followed using the same parameters as in Subheading 3.1.3, but the fluorescence emission is read every 5 min during the time required to complete the refolding process (*see* **Note 18**). In order to homogenize the samples, these are briefly shaken (for 30 s) before each determination.

3.1.5 End-Point Amyloid Disaggregation Assay

The search for compounds with the capacity to disaggregate amyloid aggregates, as well as to disrupt preformed amyloid oligomers and fibrils, may be crucial to effectively treat conformational diseases. Since IBs can be considered as reservoirs of amyloid-prone proteins in their amyloid conformation (from oligomers to mature fibrils), purified IBs (as described in Subheading 3.1.2.) may be used, without any additional treatment, in a fast and straightforward procedure to screen large libraries of disaggregating compounds.

1. Centrifuge 15 μL of purified IBs at $OD_{360} = 10$ for 10 min at 12,000 *g*.
2. Resuspend the pellet with 890 μL of PBS, 100 μL of Th-T (final concentration of 25 μM), and 10 μL of the compound according to the different disaggregation assay.
3. Prepare the appropriate controls. The positive control (maximum fluorescence) is the resuspended pellet with the same buffer without any compound (add 10 μL of compound solvent), the negative control (minimum fluorescence) is PBS with ThT (add 10 μL of compound solvent), and the compound control is that without Th-T (increase the PBS volume to 100 μL).
4. The Th-T fluorescence is measured in triplicate in the same way as that described in **step 5**, Subheading 3.1.3.

3.1.6 Kinetic Amyloid Disaggregation

Follow the same steps as those described in Subheading 3.1.5. The fluorescence emission is recorded every 5 min during the time required to complete the refolding process (*see* **Note 18**). In order to homogenize the samples, these are briefly shaken (for 30 s) before each determination.

3.2 In Cellulo Methods

Because in vitro conditions are usually far from the physiological ones, in the present section, we address the use of prokaryotic systems (bacteria) to monitor the amyloid aggregation process in vivo. The proposed in cellulo methods allow to study the amyloid aggregation in living organisms wherein, in contrast to the in vitro assays, amyloid-prone proteins and drugs can interact with other cellular components. In all the in cellulo methods described below, cell growth must always be considered as this can vary between induced and non-induced controls and in the presence or absence of compounds. To do so, it is important to determine the absorbance at 600 nm and to correct the fluorescence obtained as a function of cell growth. In addition, it should be considered that some compounds can show certain cellular toxicity, which may affect the bacterial growth. Comparison of inhibition data obtained in vitro and in vivo can give information about the ability of the compounds to cross cell membranes; hence, a high inhibition in vitro but low inhibition in cellulo may be related to a limited capacity of the compound to cross cell membranes.

3.2.1 End-Point In Cellulo Amyloid Aggregation Assay (Fig. 3)

1. Day 1: Prepare the overnight culture inoculating 10 mL of M9 minimal medium (*see* **Note 19**) containing 10 μL of the appropriate antibiotic and 10 μL of Th-S (*see* **Note 20**) with a single colony of bacterial cells bearing the plasmid to be expressed. Incubate at 37 °C under agitation.
2. Day 2: All the samples are prepared in sterilized Eppendorfs. Make triplicates for each sample.
   (a) Negative control of each compound: 200 μL of overnight culture are used to inoculate 790 μL of fresh M9 minimal medium containing 1 μL of antibiotic and Th-S and 10 μL of the compound. These samples are negative controls, as the protein expression has not been induced with IPTG (*see* **Note 21**).

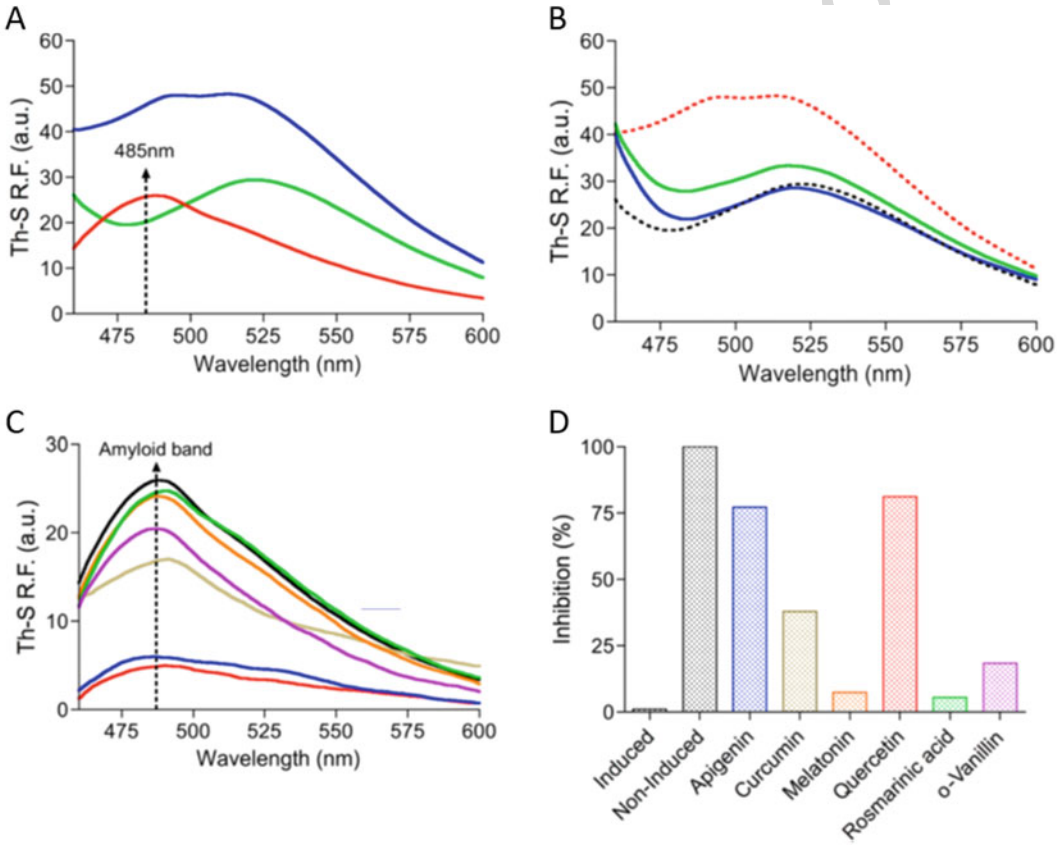

Fig. 3 Th-S staining of induced and non-induced bacterial cells. (**a**) Th-S fluorescence spectra of bacterial cells overexpressing Aβ42 (blue), non-induced bacterial cells (green), and amyloid band obtained by subtraction of the for non-induced cells from that of induced cells (red). (**b**) Th-S spectra of induced and non-induced bacterial cells grown in the presence of inhibitor; induced (green lines) and non-induced (blue lines) cells in the presence of inhibitor, induced (dashed red lines) and non-induced (dashed black lines) cells without inhibitor. (**c**) Amyloid band in the presence or absence of different inhibitors. (**d**) Inhibition percentage of Aβ42 aggregation in the absence or presence of different inhibitors. (Modified with permission from [36])

(b) Prepare the samples with the different compounds to be tested in the same way as above adding 1 μL of IPTG in the medium.

(c) To make the positive control, induced bacterial cells are used without any compound. A volume of 10 μL of the solvent compound are added instead of free compound. These samples display the maximal potential aggregation of the protein in bacteria.

(d) As a negative control, non-induced cells are used without compound, showing the minimal expression of the amyloid protein. A volume of 10 μL of the solvent compound are added instead of the compound.

3. Incubate all the samples overnight at 37 °C under agitation.
4. Day 3: The samples are transferred into 96-well plates (200 μL in each well and 3 wells per sample). The fluorescence is recorded with a plate reader or using a fluorimeter, using excitation and emission wavelengths of 445 nm and 485 nm, respectively.

3.2.2 *In Cellulo Amyloid Aggregation Kinetics*

When amyloid-prone proteins are overexpressed in bacteria, the amyloid aggregation can be tracked in vivo (in cellulo) and in real time. Amyloid aggregation kinetics can provide additional information at the end-point analysis of the process. Thus, using bacteria as a simple prokaryotic model, such assays may provide interesting information in real time, like the nucleation and elongation constants, so as the lag, half, and final times. In addition, this type of experiments can allow to obtain information about when and on which species an inhibitor is acting, thus helping to unravel possible mechanisms of action.

1. Follow the same procedure as that described in **steps 1** and **2** of Subheading 3.2.1.
2. The samples are transferred into 96-well plates (200 μL in each well and three wells per sample). The fluorescence emission and absorbance are recorded with a plate reader. The fluorescence is read using excitation and emission wavelengths of 445 nm and 485 nm, respectively, and the absorbance is measured at 600 nm (using the appropriate filters). All data are recorded every 5 min during the time required to complete the aggregation process (*see* **Note 18**). During all the aggregation kinetics, the samples are incubated, under sterile conditions, at 37 °C with orbital agitation at 250 rpm.

Instead of a plate reader, a fluorimeter and spectrophotometer can be used with some changes:

1. Prepare the overnight culture (*see* **step 1**, Subheading 3.2.1.).
2. For the preparation of the samples and pertinent controls, follow the same protocol as that found in **step 2**, Subheading 3.2.1. but increasing the final volume of the cell cultures from 1 to 10 mL. Incubate the cell cultures at 37 °C at 250 rpm.
3. Then, 200 μL of sample are collected every 30 min during all the time course of the kinetics to determine the fluorescence and absorbance.

3.2.3 End-Point In Cellulo Amyloid Disaggregation

A simple procedure to evaluate the amyloid-disaggregation ability of a potential drug may involve the tracking/assessment of the number of amyloid-like species in bacteria.

1. Day 1: Prepare the overnight culture inoculating 10 mL of M9 minimal medium containing 10 μL of the appropriate antibiotic with a single colony of bacterial cells. Incubate at 37 °C under agitation.
2. Day 2: Start the protein production (*see* Subheading 3.1.1) but inducing 4 h instead of 16 h. Repeat the process without adding IPTG for the non-induced controls (negative controls).
3. Sample preparation: All the samples are prepared in sterilized conical tubes. Make triplicates of each sample.
   (a) Negative control of each compound: take 989 μL of not induced culture and add 1 μL of Th-S and 10 μL of the compound. These samples are the negative controls, as the protein expression has not been induced (*see* **Note 21**).
   (b) To prepare the samples with the different compounds, take 989 μL of induced cultures and add 1 μL of Th-S and 10 μL of the compound.
   (c) Positive control (maximum fluorescence): take 989 μL of induced bacterial culture and add 1 μL of Th-S and 10 μL of solvent compound. These samples display the maximal potential aggregation of the protein in bacteria.
   (d) Negative control (minimum fluorescence): non-induced cells were used without compound, showing the minimal expression of the amyloid protein. In the samples without the compound, 10 μL of the solvent compound had to be added to 989 μL of non-induced cells and 1 μL of Th-S.
4. Incubate all the samples at 37 °C under agitation until day 3.

5. Day 3. Transfer the samples into 96-well plates (200 μL in each well, three wells per sample). The fluorescence and absorbance are read using a plate reader, applying excitation and emission wavelengths of 445 nm and 485 nm, respectively, and OD at 600 nm. Alternatively, the fluorescence emission can be followed with a fluorimeter using the same excitation and emission wavelengths and with a spectrophotometer to determine the absorbance at 600 nm.

3.2.4 *In Cellulo* Amyloid Disaggregation Kinetics

This assay can provide detailed information about the amyloid disaggregation process, allowing to determine not only the final amyloid reduction but also the time required to disaggregate IBs and the effect of the drug concentration on the process.

1. Follow the same procedure as that described in **steps 1**, **2**, and **3** of Subheading 3.2.3.
2. The samples are transferred into 96-well plates (200 μL in each well, three wells per sample). The fluorescence emission is recorded using excitation and emission wavelengths of 445 nm and 485 nm, respectively, and the absorbance is determined at 600 nm. All data are read every 5 min (*see* **Note 18**). During the kinetic measurements of disaggregation, the samples are incubated at 37 °C under orbital agitation at 250 rpm.

Instead of a plate reader, a fluorimeter can be used with some changes.

1. Prepare the cultures as described in **steps 1** and **2** of Subheading 3.2.3.
2. To prepare the samples and pertinent controls, follow the same protocol as that described in **step 3** of Subheading 3.2.3. but increasing the final volume of the cell cultures from 1 to 10 mL. Incubate the cell cultures at 37 °C at 250 rpm.
3. Then, 200 μL of the sample are collected every 10 min during of the whole disaggregation process to determine the fluorescence and absorbance using a fluorimeter and UV-Vis spectrophotometer, respectively.

3.3 Amyloid-Prone Proteins Fused to Fluorescent Proteins

Although the use of specific amyloid dyes such as Th-T/-S and CR are the most employed techniques for the observation and monitoring of protein aggregation, there are other techniques that allow to achieve the same goal and which consist in constructing a fusion between the amyloid-prone protein and a fluorescent protein that acts as a reporter protein.

This assay exploits the kinetic competition between amyloid-prone protein aggregation and fluorescent protein folding. Given that the aggregation rate of the fluorescent protein is constant, the aggregation rate of the amyloid-prone protein can affect the normal

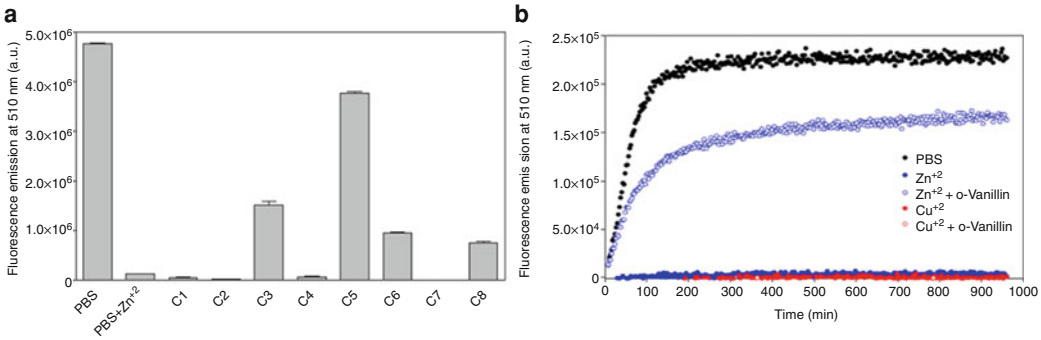

Fig. 4 Recovery of the Aβ42-GFP fluorescence in the presence of chemical compounds. (**a**) Aβ42wt-GFP IBs were denatured in 8 M Gdn and diluted 100-fold in PBS in the absence or presence of 25 μM Zn^{2+} and in the presence of small chemical compounds at a final concentration of 25 μM. (**b**) GFP fluorescence recovery kinetics upon dilution of denatured Aβ42wt-GFP IBs in PBS (black solid circles) and PBS with 25 μM Cu^{2+} (red) or Zn^{2+} (blue) ions, in the absence (solid circles) or presence (empty circles) of 25 μM *o*-vanillin. (Modified with permission from [31])

folding of the reporter protein [31]. A fast amyloid aggregation impedes the correct folding of the fluorescent protein, whereas the blocking or decrease of amyloid aggregation favors the correct folding of the fluorescent protein [31]. Consequently, protein aggregation may be related to the fluorescence of the reporter protein, a higher fluorescence indicating lower amyloid aggregation and vice versa (Fig. 4).

In this case, staining dyes are not necessary, and protein aggregation can be followed by tracking the reporter protein fluorescence. One of the most used fluorescent proteins is the green fluorescent protein (GFP), which displays excitation and emission wavelengths of 405 nm and 510 nm, respectively. All the above-mentioned procedures can be used by monitoring the emission of the fluorescent protein.

3.4 Detection of Protein Aggregation

This section describes the different techniques used to detect or track amyloid aggregation, apart from those discussed above.

Optical Fluorescence and Confocal Microscopy

In cellulo Th-S binding can be easily monitored using both optical and confocal microscopes. Figure 5 shows that induced bacterial cells display high fluorescence because of Th-S staining or how the use of fluorescent reporter proteins allows to easily detect IBs. These techniques allow to observe that the fluorescence can drastically be reduced in the presence of active inhibitors. Both optical and confocal microscopies can be applied to cell samples (in cellulo methods) and purified/isolated IBs (in vitro methods).

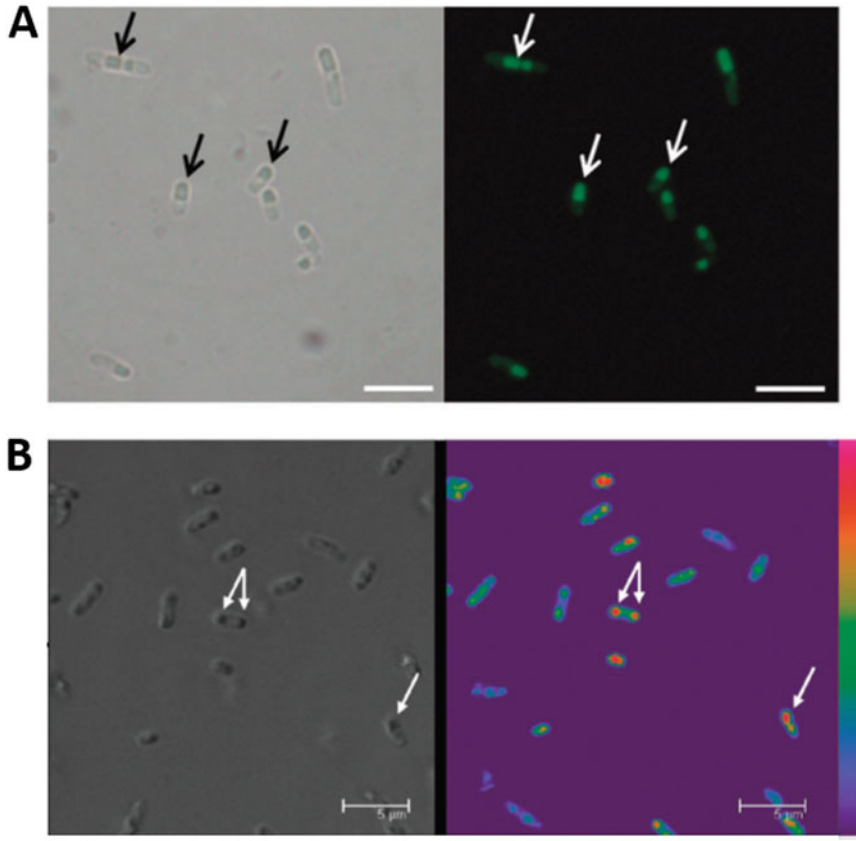

Fig. 5 Optical fluorescence and confocal microscopy images of bacterial cells. The arrows indicate the position of the IBs. Scale bars correspond to 5 μm. (**a**) Optical fluorescence microscopy images of bacterial cells expressing SPC-SH3 mutants stained with Th-S. The left and right panels correspond to phase-contrast microscopy and fluorescence microscopy under UV light, respectively. (**b**) The left and right panels correspond to the phase-contrast and confocal laser microscopy image, respectively. Image color code with intensity LUT (Pcolor4) in which purple was used to encode the background, and blue, green, and red to encode the increasing Th-S fluorescence. (Adapted with permission from [37])

3.4.1 Optical Fluorescence Microscopy

1. Follow the steps described in the in vitro methods Subheadings 3.1.3. and 3.1.5. for the end-point amyloid aggregation (**steps 1–4**) and disaggregation (**steps 1–3**) studies, respectively, or described in the in cellulo methods Subheadings 3.2.1. and 3.2.3. for the end-point amyloid aggregation and disaggregation studies, respectively (until day 3). So far, it is not necessary to add Th-T or Th-S since the staining can be done later.
2. The cells and IBs are collected by centrifugation, at 3500 *g* for 5 min, and washed with PBS buffer.
3. The samples are incubated for 1 h in the presence of 125 μM of Th-S (*see* **Note 22**).
4. The samples (bacterial cells or IBs) are pelleted by centrifugation, at 3500 *g* for 5 min, and resuspended in PBS (*see* **Note 23**).

5. 10 μL of the sample are deposited on top of glass slides.
6. The images are obtained under UV light using the correct filters for the excitation and emission or using phase-contrast microscopy.

3.4.2 Confocal Microscopy

1. End-point confocal microscopy.
   1. Follow the same procedure as that described in Subheading 3.4.1.
   2. In this case, the images are obtained with the appropriate excitation and emission wavelengths depending on whether Th-S or amyloid-prone proteins fused to fluorescent proteins are used.
2. Time-lapse confocal microscopy.
   1. Immediately after inducing protein expression, 10 μL of cell culture are placed on top of sterile microscopy slides coated with a layer of solid medium (supplemented with 1 mM IPTG and 2% agarose).
   2. The slides are covered with coverslips, and culture growth is monitored at 37 °C using a confocal laser scanning microscope equipped with a temperature-controlled incubation chamber.
   3. The fluorescence images are obtained at specified time intervals, exciting the samples and recording the emission at the corresponding wavelengths.
   4. The images are digitally processed, in order to track the amyloid aggregation, the fluorescence in the emergent IBs, and the fluorescence of bacterial cytosol; quantification is achieved using the appropriate software (*see* **Note 24**).

Flow Cytometry

Flow cytometry (FC) represents a fast, quantitative, and noninvasive method to detect the presence of IBs in bacteria (at the single-cell level) by fluorescence. This technique may be applied for the analysis of the impact of chemical compounds on aggregation.

1. Proceed in the same way as explained in Subheading 3.2.1. or 3.2.3. for end-point in cellulo amyloid aggregation or amyloid disaggregation studies, respectively, until day 3, changing the Th-S concentration from 25 μM to 125 μM.
2. Day 3: check the absorbance at 600 nm of each culture and dilute them at an $OD_{600\ nm}$ of 0.05 in PBS, maintaining a final Th-S concentration of 125 μM.
3. Optimize the fraction of single cells in the samples by gentle sonication.
4. Use an instrument equipped with a 355 nm-UV laser.

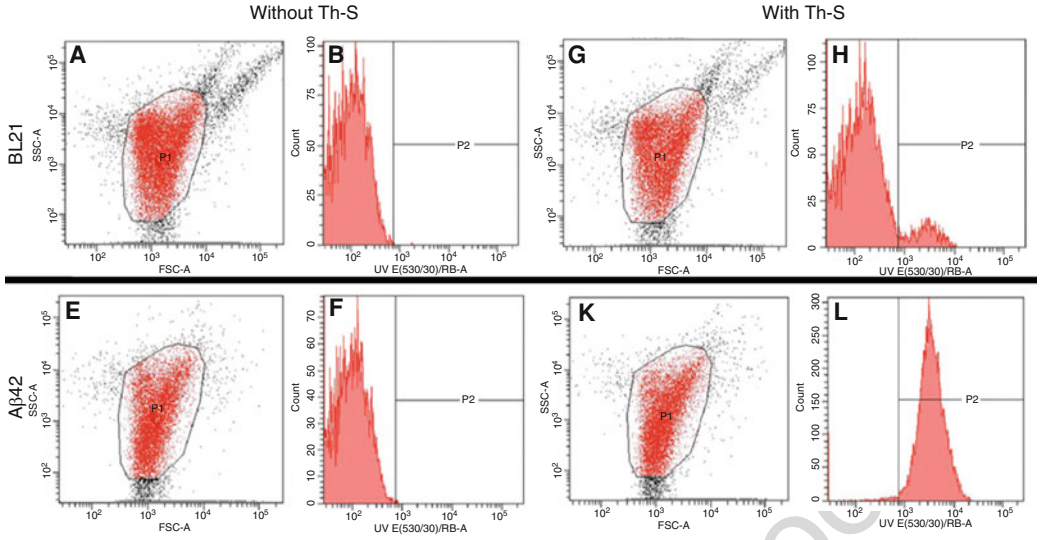

Fig. 6 Detection of cells containing amyloid-like IBs using Th-S and flow cytometry. The left and right panels correspond to the analysis in the absence and presence of Th-S, respectively. Panel **a** corresponds to forward scatter (FSC) versus side scatter (SSC) dot-plots showing the P1 gate. The cells in P1 were analyzed by fluorescence emission at 530 nm upon excitation at 355 nm, and the population P2 corresponds to cells exhibiting fluorescence emission. Panels **b**, **d**, **f**, and **h** correspond to cell frequency histograms. Non-induced BL21 cells (**a**, **b**, **e**, and **f**) and BL21 cells expressing Aβ42 (**c**, **d**, **g**, and **h**). (Adapted with permission from [37])

5. The cells are gated (P1) by forward scatter (FSC) and side scatter (SCC) signals. The cells in P1 are analyzed through the yellow/green fluorescence emission of Th-S (Fig. 6). Data analysis is performed with the appropriate software.

3.5 Secondary Structure Analysis

It is widely accepted that amyloid structures inside bacteria are reorganized into β-sheet-rich aggregates in IBs [5, 6]. Since these β-sheet-rich structures exhibit specific circular dichroism (CD) and Fourier transform infrared (FT-IR) spectra, as well as X-ray diffraction patterns, these techniques have become standard assays for amyloid detection. Hence, secondary structures can be tracked via the increment of β-sheet structures detected by circular CD or FT-IR. The techniques described below can only be applied for the study of in vitro aggregation.

3.5.1 Circular Dichroism (CD)

The β-sheet secondary structure in amyloids displays a characteristic minimum at 217 nm in the far-UV region of the CD spectrum.

1. Purified IBs are placed at the required concentration (usually ranging from 5 to 20 μM) in a quartz cell of 0.1 cm (or 1 cm) path length.
2. The CD spectra are usually recorded from 190 to 250 nm at room temperature, at a spectral resolution of 1 cm^{-1} and a scan rate of 15 nm/min using a spectropolarimeter.

3. In order to determine the secondary structure components of each sample, the CD spectra can be deconvoluted with the K2D2 Suite (http://www.ogic.ca/projects/k2d2/) or similar programs (*see* **Notes 25** and **26**).

3.5.2 Fourier Transform Infrared Spectroscopy (FTIR)

The intermolecular β-strands in the amyloid core display a characteristic band at 1620–1630 cm^{-1} in the amide I region of the infrared spectrum. In addition, a secondary band at ~1692 cm^{-1} can be detected, which is assigned to antiparallel β-sheet conformation. This secondary band corresponds to the splitting of the main band and cannot be considered as conclusive for a β-sheet antiparallel conformation. Solution absorption FTIR is a common technique to analyze secondary structures of proteins. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR FT-IR) is a different FTIR sampling technique that is becoming popular because aggregates (which tend to precipitate) can be deposited onto an ATR crystal and be analyzed in the solid state. A basic and simplified method used for the analysis of secondary structure contents of protein assemblies is described below.

Absorption FT-IR Spectroscopy Analysis

1. Air-dry or lyophilize the samples.
2. Exchangeable hydrogen atoms may be replaced by deuterium by dissolving the dried proteins in D_2O.
3. The protein samples are inserted between CaF_2 windows using a 50 mm Mylar spacer.
4. Infrared spectra are recorded with an FT-IR spectrophotometer equipped with a liquid nitrogen-cooled mercury/cadmium telluride detector, purged with a continuous flow of dinitrogen gas.
5. About 250 interferograms are usually recorded at room temperature with a spatial resolution of 1–2 cm^{-1}.
6. For each single spectrum, the background water vapor is subtracted, and the baseline is corrected.
7. The area of the spectrum between 1700 and 1600 cm^{-1} is normalized by fitting it through overlapping of the Gaussian curves. The amplitude, center, and bandwidth at half of the maximum amplitude and the area of each Gaussian function are calculated using of a nonlinear peak-fitting program.
8. Second derivatives of the spectra can be used to determine the frequencies at which the different spectral components are located.

ATR FTIR Spectroscopy Analysis

1. The samples do not require previous manipulation except if potential interference of the buffer is suspected. In this case, sample centrifugation and buffer replacement with milliQ water are recommended (two or three washing repetitions).
2. 5–10 μL of the sample are placed in a FTIR spectrometer with an ATR accessory.
3. Dry the samples gently with N_2 or argon.
4. The final spectrum consists of the accumulation of at least 64 independent scans, measured at a spectral resolution of 1 cm^{-1} over the 1700–1600 cm^{-1} range.
5. For each final spectrum, the background water vapor is subtracted, and the baseline is corrected.
6. Second derivatives of the spectra can be used to determine the frequencies at which the different spectral components are located.
7. Additionally, the infrared spectra can be fitted through overlapping of the Gaussian curves, and the amplitude, center, and bandwidth at half of the maximum amplitude and area of each Gaussian function can be calculated using a nonlinear peak-fitting program.

4 Notes

1. Approximately 1 mL of 1 M NaOH.
2. IPTG is used to induce *Escherichia coli* protein expression, the gene being under the control of the *lactose* operon (*lac* operon). Other promoters, where induction conditions are different, can be considered as the *araBAD* promoter, induced by adding L-arabinose or P_L promoter by shifting the temperature from 37 to 42 °C.
3. To start adjusting the pH, concentrated HCl (12 N) can be used to obtain a pH below but close to 8. To finish adjusting and to avoid a sudden change in pH, less concentrated HCl is used.
4. Due to its low solubility in water, PMSF must be dissolved in ethanol or methanol.
5. Mg^{2+} ions are essential for DNase activation.
6. Th-S is a mixture of compounds that results from the methylation of dehydrothiotoluidine with sulfonic acid; hence, its molar concentration cannot be accurately calculated.

7. The maximum solubility of guanidine hydrochloride in water at room temperature is around 6 M. To prepare a 8 M solution, it is necessary to heat the solution at 35–40 °C for about 30 min.
8. Guanidine hydrochloride 8 M is likely to precipitate at room temperature. Before using it, heat the solution to 37 °C until complete dissolution.
9. Sugars like glucose cannot be sterilized by autoclaving because of the Maillard reaction (caramelization).
10. The overnight culture should usually be inoculated with an isolated colony from a freshly streaked plate. Alternatively, glycerol stock solution can be used; bacterial glycerol stocks are convenient for long-term storage. Bacteria on LB agar plate can be stored at 4 °C for a few weeks. The addition of glycerol stabilizes frozen bacteria, preventing cell membrane damages and therefore keeping the cells alive. Glycerol stocks of bacteria can be stored at −80 °C for several years. Mix 500 μL of overnight culture and 500 μL of 50% glycerol (final concentration of 25%) in a 2 mL screw top vial or cryotube and gently mix. Freeze at −80 °C.
11. If the bacteria grow too dense, the cells will inhibit the protein expression.
12. The steps described allow the separation of the IBs from the rest of the bacterial material, such as the phospholipids of the cell membrane, nucleic acids, proteins, etc.
13. Instead of Th-T, CR can be used. The absorbance of CR shifts from orange-red to pink in the presence of intermolecular β-sheet structures.

    UV-Vis absorbance of CR: Spectra can be recorded in the range 375–675 nm using a quartz cuvette of 1 cm optical length. Usually, the presence of amyloid aggregates promotes significant light scattering, which must be subtracted from the resulting CR spectra. Incubate the sample with CR (5–20 μM) for 10–15 min before the measurements. To detect the typical amyloid band at ~541 nm, the differential CR spectrum is plotted by subtracting the spectrum of free CR from that of bound CR.

    CR birefringence assay: Birefringence is the decomposition of a ray of light into two rays when it passes through certain anisotropic materials. The binding of CR molecules to amyloid fibrils axis usually causes apple-green birefringence when viewed through cross-polarized light. This assay provides a more specific assessment of the amyloid nature of the protein aggregates than CR absorbance measurements. Samples are incubated for 1 h with 50 μM CR. The samples are centrifuged at 14,000 *g* for 5 min, and the aggregated fraction is resuspended with milliQ water (repeat three times). The

precipitated fraction is placed on a microscope slide, and the CR birefringence can be detected under cross-polarized light using an optical microscope.

14. Add 10 μL of the solvent in which the compounds are dissolved, 10 μL of denatured IBs, 880 μL of PBS, and 100 μL of Th-T (final concentration of 25 μM).
15. Add 10 μL of the denaturing agent used, 10 μL of the compound solvent, 880 μL of PBS, and 100 μL of Th-T (final concentration of 25 μM).
16. Compound control is needed to take into account the possible fluorescence of each compound. 10 μL of denatured IBs, 980 μL of PBS, and 10 μL of the compound.
17. Amyloid aggregation in bacteria follows a nucleation-polymerization mechanism in which three phases can be distinguished: (1) nucleation or lag phase, where the soluble protein associates to form nuclei in a thermodynamically disfavored phase; (2) elongation reaction, where the preformed nuclei act as seeding soluble species that are added to pre-existing fibril templates, in a favorable thermodynamic process; and (3) plateau phase. For a detailed description of the aggregation kinetics, the determination of the nucleation and elongation parameters is essential [32, 33]. The kinetic constants can be derived from time-course experiments exploiting the fact that the aggregation process of the soluble protein into amyloid can be described as an autocatalytic reaction [33].
18. Stop the kinetics when the emission fluorescence stabilizes (does not increase) for about 20 min.
19. Culture media such as LB and 2×YT medium can dramatically interfere with the Th-S fluorescence signal; therefore, M9 minimal medium is used with Th-S.
20. For in cellulo studies, Th-S is used instead of Th-T because it is a dye capable of crossing bacterial membranes [34].
21. The negative control of each compound is needed to discard any potential effects of the compound on the Th-S signal (some compounds may display fluorescence in the Th-S region).
22. When using a protein reporter (such as GFP), this step is not necessary.
23. This step is important in order to avoid the residual Th-S fluorescence.
24. Amyloid aggregation in IBs can be tracked in real time by time-lapse confocal microscopy. In this case, it is necessary to determine the IBs fluorescence per IBs area from the confocal microscopy images using the appropriate software. The data

obtained are subsequently normalized, 0 being the residual fluorescence before induction and 1 the IBs' fluorescence at the end point of the kinetics of each particular variant. Finally, the normalized fluorescence data are fitted using the classical nucleation-polymerization model (*see* **Note 17**).

25. To study the aggregation kinetics, a fixed wavelength of 217 nm can be used to monitor the increase or decrease in β-sheet contents.
26. To avoid the presence of noise or background in the spectra, buffers with high ionic strengths or chiral molecules should be avoided.

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