Thioflavin-S Staining of Bacterial Inclusion Bodies for the Fast, Simple, and Inexpensive Screening of Amyloid Aggregation Inhibitors

Abstract: Amyloid aggregation is linked to a large number of human disorders, from neurodegenerative diseases as Alzheimer's disease (AD) or spongiform encephalopathies to non-neuropathic localized diseases as type II diabetes and cataracts. Because the formation of insoluble inclusion bodies (IBs) during recombinant protein production in bacteria has been recently shown to share mechanistic features with amyloid self-assembly, bacteria have emerged as a tool to study amyloid aggregation. Herein we present a fast, simple, inexpensive and quantitative method for the screening of potential anti-aggregating drugs. This method is based on monitoring the changes in the binding of thioflavin-S to intracellular IBs in intact *Eschericchia coli* cells in the presence of small chemical compounds. This *in vivo* technique fairly recapitulates previous *in vitro* data. Here we mainly use the Alzheimer's related β-amyloid peptide as a model system, but the technique can be easily implemented for screening inhibitors relevant for other conformational diseases simply by changing the recombinant amyloid protein target. Indeed, we show that this methodology can be also applied to the evaluation of inhibitors of the aggregation of tau protein, another amyloidogenic protein with a key role in AD.

Keywords: Amyloid formation, Alzheimer's disease, Bacterial inclusion bodies, Beta-amyloid peptide, Conformational diseases, Tau protein, Thioflavin-S

Short "running title": Simple Screening of Aβ42 and Tau Aggregation Inhibitors in Intact *E. coli* Cells

1. INTRODUCTION

Amyloid aggregation is a process by which some monomeric peptides or proteins undergo a conformational change into misfolded species that expose specific amyloid-prone sequences usually increasing hydrophobic surfaces, otherwise generally buried in folded structures. This increases intermolecular attractive forces favoring the formation of steric zipper (two self-complementary β-sheets that form the spine of an amyloid fibril), rendering them prone to self-associate into increasingly ordered and insoluble oligomeric and polymeric species or fibrils [1-3]. Amyloidogenic proteins, despite having distinct amino acid composition, length, and *in vivo* distribution, eventually lead to similar highly ordered aggregated structures. Amyloid structures have a core region formed by repetitive arrays of β-sheets oriented parallel to the fibril axis [1,4,5], and display similar biochemical, biophysical, tinctorial and morphological properties [6]. Even though amyloid aggregates can play specific physiological roles [1,7], in most cases they seem to be the root cause of a number of human diseases, the so-called amyloidoses, conformational diseases or protein misfolding disorders [1,6]. These include a broad range of disorders, from neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases or transmissible sporadic encephalopathies to non-neurodegenerative systemic and localized amyloidoses such as lysozyme and fibrinogen amyloidosis or type II diabetes and cataracts [8].

Alzheimer's disease (AD) is likely the conformational disease where more intensive research has been devoted to understand the mechanisms underlying amyloid aggregation, as well as to pinpoint its role in AD neuropathogenesis and as a target for anti-Alzheimer drug discovery. The specific amyloidogenic protein involved in AD is the β-amyloid peptide (Aβ). Aβ is a peptide from 38 to 43 amino acids that arises from the proteolytic cleavage of the amyloid precursor protein (APP). Its most common form is 40 residues in length (Aβ40) whereas the most prone to aggregate and neurotoxic form is that of 42 residues (Aβ42). According to the most widely accepted theory about the etiology of AD, the "amyloid cascade hypothesis", an increased formation of Aβ and its subsequent aggregation and deposition in neural tissue are the culprits of the neurodegeneration in AD patients [9-12]. Aβ formation and aggregation are thought to trigger a cascade of deleterious events that eventually result in neuronal dysfunction and death and dementia. Thus, the discovery of inhibitors of Aβ aggregation is an area of very active research [2,6,13-16], together with that of other Aβ-directed drug candidates aimed at reducing the levels of Aβ in brain by inhibiting its formation (inhibitors of β- and γ-secretases) or by increasing its clearance (active or

passive immunotherapy), in the search for effective medications that can delay the onset of AD and slow or halt its progression [16,17].

Usually, the screening of inhibitors of Aβ aggregation is carried out *in vitro* using expensive synthetic peptides and thioflavin-T (Th-T)-based fluorometric assays involving a variety of experimental conditions [18-22]. Even *in vitro*, Aβ aggregation itself is a difficult process to study, inasmuch as it is highly sensitive to a number of factors including purity and experimental conditions such as solvents or buffers used or mixing conditions [16]. Moreover, in general, *in vitro* evaluation of compounds against isolated protein targets is increasingly perceived as being too far from the (patho)physiological conditions where drugs have to act, with implications regarding the relevance or reliability of the obtained *in vitro* activity results when translated to the *in vivo* context. Indeed, in living organisms proteins are prone to associate and interact with other cellular components, giving rise to complex interaction networks [23,24] that are absent in *in vitro* tests [25]. Thus, *in vivo* Aβ aggregation is dependent on a number of factors that define its complex cellular environment [1,26,27], which cannot be captured in *in vitro* assays. Indeed, it has been suggested that Aβ aggregation can proceed through different pathways *in vitro* and *in vivo* thereby leading to different Aβ aggregates [12].

The study of Aβ aggregation and the screening of Aβ anti-aggregating hit or lead compounds would greatly benefit from novel phenotypic assays that can recapitulate key aspects of the (patho)physiological process of Aβ aggregation that are absent in *in vitro* assays [25], and that can be more cost-effective and simpler than classical *in vivo* assays in animal models.

In this light, bacteria have emerged as suitable models to monitor protein aggregation [27,28]. Protein aggregation also occurs during the production of heterologous proteins in prokaryotic systems, giving rise to insoluble protein aggregates, the so-called inclusion bodies (IBs), which limits the application of bacteria for recombinant protein production in the biotechnology industry [29]. Contrary to the initial assumption that IBs consisted of disorderly deposited inactive proteins, the existence of highly ordered amyloid-like structures inside IBs has been recently demonstrated [30-33]. This has dramatically shifted the consideration of IBs from being regarded as useless "molecular dust-balls" to being considered as an excellent and simple but biologically relevant model to study the mechanisms of amyloid folding and deposition related to conformational diseases. Indeed, Wang *et al.* have recently reported that amyloid structure could be an inherent property of all heterologous proteins overexpressed in IBs [31]. In contrast to Aβ, which is an intrinsically unstructured protein, the polypeptides used in the study of Wang *et al.*

[31] are globular and not associated with any disease, indicating that the formation of amyloid-like structures inside IBs might be a general phenomenon. Therefore, it seems that the establishment of an inter-backbone, hydrogen-bonded network that stabilizes related fibrillar structures enriched in the β-sheet conformation is a common force driving protein aggregation in the cell.

Different methodologies can be used to monitor protein aggregation in bacteria, which involve the fluorescence detection of either genetically encoded fusion tags such as the green fluorescent protein (GFP) or conformational-sensitive fluorescent dyes, prominently thioflavin-S (Th-S) [27]. Regarding the first methodology, we have very recently developed a new method for the detection of inhibitors of metal-promoted Aβ42 aggregation using purified bacterial IBs formed by an Aβ42-GFP fusion protein in *Escherichia coli* [34]. In this method, IBs fluorescence correlated with the aggregation level of Aβ42, as a result of the kinetic competition between the folding of the GFP domain, which leads to increased fluorescence, and the aggregation of Aβ42 moiety, which causes the entire fusion protein to misfold thereby precluding the folding of the GFP domain and leading to decreased fluorescence [34]. In the presence of Aβ42 aggregation inhibitors, GFP can fold into its native structure, giving rise to the fluorescent signal. The main drawback of this method is that any factor that can affect GFP folding or aggregation rates would lead to a biased readout, irrespective of the aggregation propensity of the protein. To overcome potential effects of the inhibitor compounds on GFP folding that might mask their anti-amyloid capacity, the presence of amyloid aggregates inside bacteria might be directly monitored by using conformational-sensitive fluorescent dyes [35]. The most commonly used amyloid specific dyes are Th-S, Th-T, and Congo Red (CR), and among them, Th-S is the most convenient for *in vivo* detection of amyloid deposits, including bacterial IBs, by virtue of its ability to cross membranes and to penetrate inside the cells without interfering with the amyloid processes. Th-S is superior to CR, which possesses intrinsic amyloid inhibition capacity [36], and to the less lipophilic P-glycoprotein substrate Th-T, which cannot conveniently internalize in bacterial cells [37]. Th-S binds to amyloid fibrils, displaying an intensity increment and a maximum shift of its fluorescence spectrum upon binding [38]. Accordingly, Th-S stained amyloid-like aggregates display specific fluorescence when excited by UV or blue light [35,39,40].

Herein, we describe a new effective method for the screening of putative amyloid aggregation inhibitors, based on the direct Th-S staining of bacterial IBs in intact *E. coli* cells. This method is simpler and cheaper than classical *in vitro* and *in vivo* assays and physiologically more relevant than the commonly

used *in vitro* tests, albeit, of course, less relevant than *in vivo* studies in animals or in humans. Moreover, this method avoids the use of reporter proteins, thus preventing potential interferences of factors affecting the folding of the reporter protein. In order to assess the applicability of the method we have determined the *in vivo* Aβ42 anti-aggregating activity in intact *E. coli* cells of a number of compounds with well-characterized *in vitro* inhibitory activity: the commercially available bioactive compounds *o*-vanillin and propidium iodide, the anti-AD drugs tacrine and donepezil, and the anti-AD investigational compounds (±)-huprine Y and heterodimers **1**-**5** (Fig. **1**). Moreover, to demonstrate that this method can be applied to other amyloidogenic proteins, we have set up an analogous methodology for the screening of inhibitors of the aggregation of tau protein and we have applied it to the evaluation of some selected compounds.

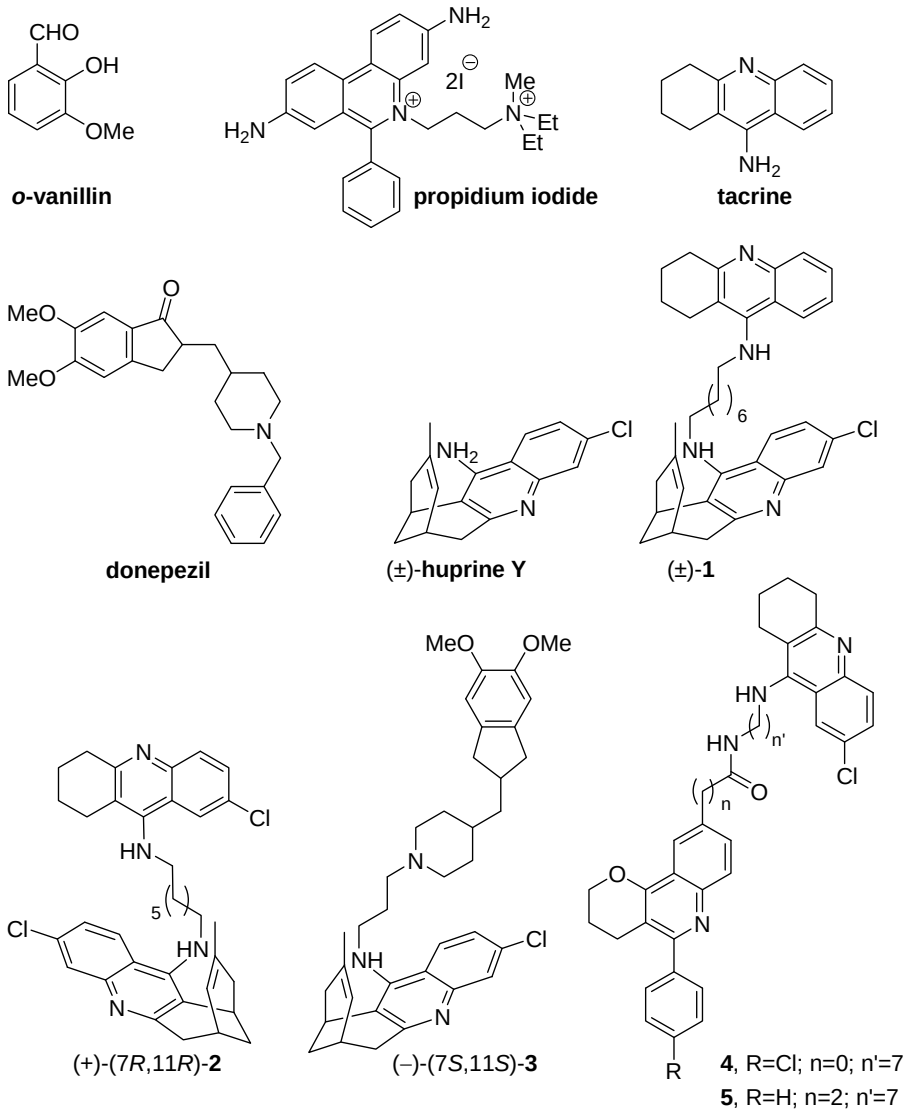

Fig. (1). Chemical structures of the tested compounds.

2. MATERIALS AND METHODS

2.1. General

Th-S (T1892) and other chemical reagents were purchased from Sigma (St. Louis, MO). (±)-Huprine Y and compounds **1-5** were prepared as previously described [41-44]. Microbiological reagents were purchased from Conda Lab. (Spain) and isopropyl β-D-thiogalactopyranoside (IPTG) from Apollo Scientifics (UK). Solutions were prepared in double-distilled water purified through a Milli-Q system (Millipore, USA). Th-S and inhibitors were solubilized in DMSO at 2.5 mM and 10 mM, respectively.

2.2. Bacterial Growth and Protein Expression

2.2.1. Bacterial Growth

E. coli competent cells BL21(DE3) were transformed with a pET28a vector (Novagen, Madison, WI) carrying the DNA sequence of Aβ42. As a non-amyloid protein control, competent cells BL21(DE3)pLysS were transformed with a pET21 vector (Novagen, Madison, WI) carrying the DNA sequence of HET-s(1-227) expressed as a C-terminal histidine-tagged construct. In order to obtain a homogenous bacterial culture stock to favour the reproducibility of the assays, 25 mL of DYT medium (tryptone, yeast extract, NaCl) with adequate antibiotic (kanamycin or ampicillin and chloramphenicol for BL21(DE3) and BL21(DE3)pLysS, respectively) were inoculated with a single colony from solid media of *E. coli* BL21 bearing each plasmid to be expressed at 37 °C. Bacterial cultures were grown up overnight at 37 ºC. Then, 12.5 mL of sterile glycerol (60%) were added obtaining a final glycerol concentration of 20%. OD_{600} were checked and the cultures were diluted in the same media (20% glycerol in DYT with the required antibiotic) at OD_{600nm} of 1. Finally, 100 µL of bacterial culture were placed in eppendorf tubes of 1.5 mL and the tubes were frozen at -80 ºC until next use.

For tau protein production, *E. coli* BL21 (DE3) competent cells were transformed with pTARA containing the RNA-polymerase gen of T7 phage (T7RP) under the control of the promoter P_{BAD}. *E. coli* BL21 (DE3) with pTARA competent cells were transformed with pRKT42 vector encoding four repetitions of tau protein in two inserts. For overnight culture preparation, 10 mL of M9 medium containing 0.5% of glucose, 50 $\mu g \cdot mL^{-1}$ of ampicillin and 12.5 $\mu g \cdot mL^{-1}$ of chloramphenicol were inoculated with a colony of BL21 (DE3) bearing the plasmids to be expressed at 37 °C. After overnight grown, the OD_{600} is usually 2 – 2.5.

2.2.2. Protein Expression

For expression of Aβ42 peptide and non-amyloid protein and controls, we added 870 µL of DYT medium

containing the required antibiotics to the previously frozen cultures (100 μL) and finally 10 μL of IPTG (at 0.1 mM), 10 μL of Th-S (at 2.5 mM) and 10 μL of DMSO (in order to use the same amount of DMSO in the absence of inhibitor). It is known that DMSO concentrations of 2% do not affect either the amyloid formation or the bacterial survival [45-46]. The samples were grown for 24 h at 37 °C and 1,400 rpm using a Thermomixer (Eppendorf, Hamburg, Germany). For non-induced controls, 10 μL of sterile double-distilled water were used in substitution of IPTG.

For expression of tau protein, 20 μL of overnight culture were transferred into eppendorf tubes of 1.5 mL containing 980 μL of M9 medium with 0.25% of arabinose, 50 $\mu g \cdot mL^{-1}$ of ampicillin, 12.5 $\mu g \cdot mL^{-1}$ of chloramphenicol, and 10 μM of each testable drug in DMSO. The samples were grown for 24 h at 37 °C and 1,400 rpm using a Thermomixer (Eppendorf, Hamburg, Germany). In the negative control (without drug) the same amount of DMSO was added in the sample.

2.3. Thioflavin-S (Th-S) Steady-State Fluorescence

Fluorescent spectral scans of Th-S were analyzed using an Aminco Bowman Series 2 luminescence spectrophotometer (Aminco-Bowman AB2, SLM Aminco, Rochester, NY). For emission and excitation scans, ranges from 390 to 600 nm and from 300 to 400 nm using excitation and emission wavelengths of 375 and 455 nm, respectively, were performed. Excitation and emission slit widths of 5 nm were used. Spectra were acquired at 1 nm intervals and 500 $nm \cdot min^{-1}$ rates.

For the fluorescence assay of bacterial cultures, the fluorescence emission of 25 μM of Th-S at 455 nm, when exciting at 375 nm, was recorded. Culture media as DYT medium can dramatically interfere with the Th-S fluorescence signal and the differences in bacterial cells concentration might also slightly affect the Th-S relative fluorescence due to both bacterial membranes staining and scattering. Thus, both the presence of controls and an accurate determination of bacterial cell concentration are necessary to attain an unbiased quantification of the effect of each inhibitor. On the one hand, the Th-S fluorescence was normalized as a function of the bacterial concentration, determined by OD_{600} using a Shimadzu UV-2401 PC UV-Vis spectrophotometer (Shimadzu, Japan). On the other hand, the normalized Th-S relative fluorescence was determined both in the presence and in the absence of protein expression (with or without IPTG). In the present work, the ratios between the normalized Th-S fluorescence of BL21(DE3) cells expressing and non-expressing the Aβ42 peptide or tau protein were considered to correspond to 100% of relative fluorescence or 0% of inhibitory activity and used as positive control. Similarly, the

relationship between induced and non-induced BL21(DE3)pLysS cells containing pET21 and pET28 empty vectors was taken as negative control, corresponding to 0% of relative fluorescence or 100% of inhibitory effect. Besides, a non-amyloid protein as HET-s(1-227) globular domain [48] that does not form IBs (data not shown), and BL21(DE3)pLysS cells without any vector were also used as additional negative controls. By proceeding in this way a fast and accurate determination of the inhibitory activity of each compound could be carried out. Since both bacterial growth and protein expression can undergo important fluctuations among samples we carried out ten independent experiments for each assay condition and used the averaged value to compare inhibitors potency.

2.4. SDS-PAGE and Western Blotting

In order to monitor the Aβ42 peptide concentration in cell cultures in the presence and absence of inhibitors, bacterial cells were pelleted by centrifugation at 14,000 rpm (5 min, 4 ºC) and frozen at -80 ºC for 2 h. Then, cell pellets from 1 mL culture were re-suspended in phosphate buffer saline (PBS) at the indicated concentrations and were analyzed by SDS-PAGE followed by Western blotting onto nitrocellulose membranes. To visualize Aβ42, membranes were blocked in milk, stained with anti-Aβ mAb 6E10 (1:2000, Covance), anti-mouse Ab-HRP and developed by using the Western Lightning ECL kit (Perkin–Elmer, Germany).

2.5. Optical Fluorescence Microscopy

For optical fluorescence microscopy analysis, cultures were collected after overnight induction and washed in PBS buffer; non-induced bacterial cultures were used as a control. Afterward, bacterial cells were incubated for 1 h in the presence of 125 μM Th-S. In order to avoid the residual Th-S fluorescence of the free dye the samples were centrifuged at 3,500×*g* for 5 min and the obtained precipitate fraction was re-suspended in PBS. 10 μL of sample was collected and deposited on top of glass slides. Images were obtained under UV light or phase-contrast using a Leica fluorescence DMBR microscope (Leica Microsystems, Mannheim, Germany).

3. RESULTS AND DISCUSSION

Th-S staining coupled to flow cytometry has been recently reported to be an excellent screening tool to monitor protein aggregation in intact *E. coli* cells, offering advantages for the analysis of biological samples over conventional single-cell measurements or approaches that rely on averaged population

properties [35]. Unfortunately, flow cytometry is not yet a readily available tool in the majority of research labs. Herein we show that Th-S relative fluorescence upon *in vivo* binding to amyloid-like proteins can be used as an alternative simple method to assess the effect of putative anti-aggregation drug candidates.

The self-assembly in β-sheet conformation of Aβ appears to play a major causative role in AD neuropathogenesis [9-12]. As discussed previously, bacterial cells can be useful systems for studying *in vivo* protein aggregation, providing a biologically relevant environment for the screening of anti-aggregation drugs [30]. Because overexpression of Aβ40 and Aβ42 in bacteria entails the formation of IBs that display amyloid-like properties [32], here we have chosen Aβ42, the most aggregation-prone and neurotoxic Aβ peptide, to test the applicability of our method.

In order to confirm the Th-S binding to intracellular aggregates we visualized Aβ42 IBs using microscopy under UV-light. As shown in Fig. **2**, Aβ42 IBs are selectively stained inside living bacterial cells yielding a bright green-apple fluorescence, displaying a maximal excitation and emission fluorescence at 375 and 455 nm, respectively. For the positive control a ratio of ~1.8 between the maximum of fluorescence of BL21(DE3) expressing and non-expressing the Aβ42 peptide was obtained. For the negative control (BL21(DE3)pLysS containing the pET21 and pET28 empty vectors and/or the same cells without any vector) a ratio of ~1 between induced and non-induced cultures was found. As expected, the non-amyloid and soluble HET-s(1-227) domain displays a ratio of ~1.1, very close to those obtained for negative controls.

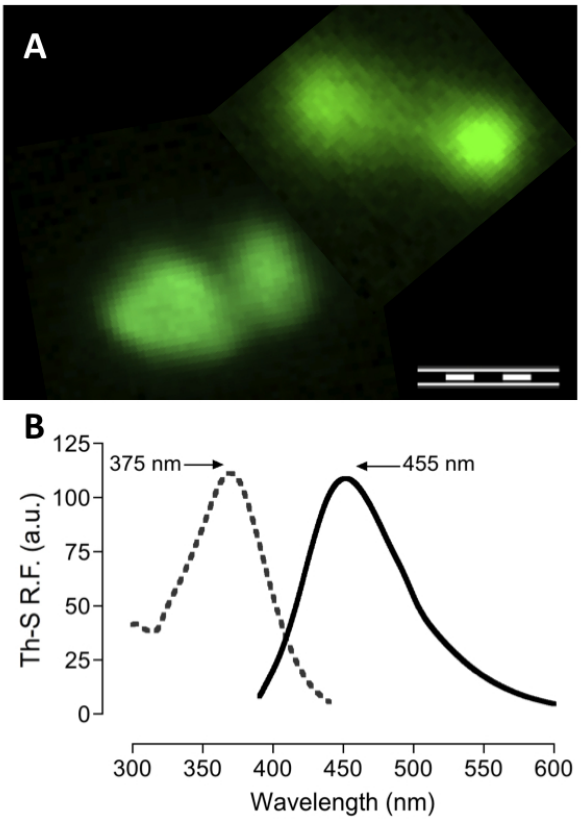

Fig. (2). Th-S staining of bacterial cells overexpressing Aβ42 peptide. (A) Optical fluorescence microscopy images of bacterial cells overexpressing Aβ42 peptide stained with Th-S. The microscopy image under UV light shows the presence of amyloid-like IBs localized at the cellular poles. Scale bars 0.5 μm. (B) Emission and excitation spectra of Th-S in the presence of bacterial cells overexpressing Aβ42 peptide. Note that excitation and emission wavelengths of 375 and 455 nm have been used, respectively.

Proceeding in the same manner with BL21(DE3) cells expressing Aβ42 in the presence of putative anti-aggregating compounds, a fast and accurate evaluation of the effect of each inhibitor can be performed. In order to discard possible adverse effects of the inhibitors on bacterial cell survival and/or Aβ42 expression, we monitored the OD_{600} of each cell culture and the Aβ42 expression level by Vis spectroscopy and western blotting, respectively. Non-induced cultures showed OD_{600} ~1.3±0.1 at the final growth time, whereas the induced ones displayed OD_{600} ~0.9±0.1 at the same incubation time. Very similar results were observed in the presence of all anti-aggregation compounds (data not shown). Moreover, western blotting assays showed that the protein expression levels were not altered by the presence of the tested compounds.

When bacterial cells overexpressing Aβ42 were incubated in the presence of 10 μM of inhibitors, for which *in vitro* Aβ42 anti-aggregating activities had been previously reported, we observed in all cases a decrease in the final Th-S relative fluorescence per cell. This is a consequence of the diminution of amyloid-like structures in bacterial cells, and therefore, of the *in vivo* inhibitory activity of the tested compounds in the intact *E. coli* cells. In Fig. **3** it can be observed that in the presence of active inhibitors, as propidium, the Th-S staining is significantly reduced whereas poorly active compounds, such as (±)-huprine Y, almost do not affect Th-S staining relative to controls (Fig. **3**, upper panel).

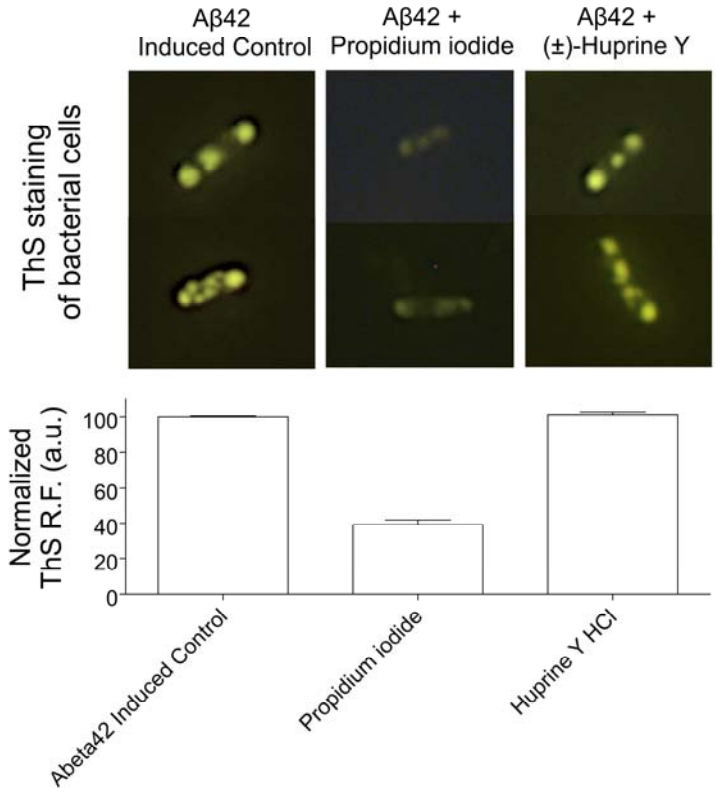

Fig. (3). Th-S staining of bacterial cells overexpressing Aβ42 peptide in the absence and in the presence of active (propidium) and inactive ((±)-huprine Y)) anti-aggregating compounds. (A) Optical fluorescence microscopy images of bacterial cells overexpressing Aβ42 peptide stained with Th-S. (B) Th-S relative fluorescence in the presence of bacterial cells overexpressing Aβ42 peptide. Note that excitation and emission wavelengths of 375 and 455 nm have been used, respectively.

Very interestingly, all tested compounds exhibited anti-aggregating activities in intact *E. coli* cells similar to those observed *in vitro* (Fig. **4** and Table **1** of Supplementary Material). Also, the order of Aβ42 anti-

aggregating potencies of the tested compounds found in the *in vivo* assays almost perfectly matches that reported *in vitro*. Thus, (±)-huprine Y, tacrine and donepezil, which had been reported to be essentially inactive *in vitro* as Aβ42 anti-aggregating compounds, display very low Aβ42 anti-aggregating activity in *E. coli*, with percentages of inhibition of 9.2%, 4.3%, and 2.5%, respectively, whereas heterodimeric compounds **1**-**3** and **5** as well as other known Aβ42 anti-aggregating compounds as propidium iodide and *o*-vanillin display significant inhibitory activities, with percentages of inhibition ranging from 23.5% to 48.5%. Heterodimer **4** exhibited a very low activity as it had been previously found *in vitro* (28.6% inhibition at 50 μM) [42].

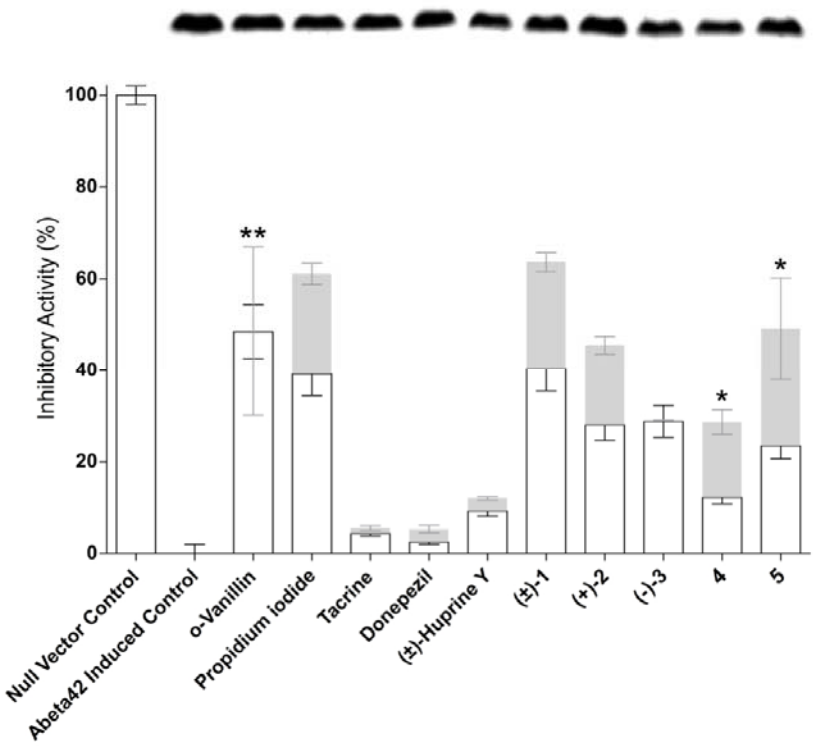

Fig. (4). Inhibitory activities of anti-aggregating compounds monitored by Th-S staining of bacterial cells overexpressing Aβ42 peptide. The graph shows the anti-aggregating effect of 10 μM of inhibitor added to bacterial culture overexpressing Aβ42 peptide after overnight incubation. The grey section in each bar denotes the reported inhibitory activity of each compound by *in vitro* determinations using a drug:Aβ42 ratio of 1:5 [42-44,49]. (∗) Note that the *in vitro* inhibitory activity of these drugs was determined using a concentration of inhibitor of 50 μM and a drug/Aβ42 ratio of 1:1 [42]. (∗∗) *In vitro* data obtained using a concentration of inhibitor of 300 μM and a drug:Aβ42 ratio of 1:0.15 [50]. In the upper part of the figure, western blotting of bacteria cells in the absence or in the presence of drugs are shown. Note that similar concentration of Aβ42 is detected in the absence and in the presence of inhibitors.

Worthy of note, all *in vivo* inhibitory activities are lower than those reported *in vitro*, with the sole exception of compound **3**, which displayed the same potency *in vivo* and *in vitro*. The drug:Aβ42 stoichiometry might explain this fact; *in vitro* assays are carried out at a ratio 1:1 or 1:5, whereas in *in vivo* studies the stoichiometry might be very different. On the one hand, we performed our assays at 10 μM of drug whereas the concentration of Aβ42 might be clearly higher. On the other hand, the intrinsic capacity of each drug to cross the bacterial membrane might restrict the inhibitor concentration in the cell. Even if the capability of a drug to cross biological membranes is a desirable property, some initial hits that are unable to cross *E. coli* membranes but active as Aβ42 aggregation inhibitors would remain undetected, and therefore lost, in the *in vivo* screen, which represents a flaw of this methodology. Finally, we decided to investigate if our protocol allowed the quantification of the anti-aggregating activity in a dose-dependent manner. In Fig. **5** we show that for compounds **4** and **5** and propidium iodide (selected because *in vitro* inhibitory activities at different drug:Aβ42 stoichiometries are available [42,49]) an increased Aβ42 anti-aggregating activity was observed for the higher drug concentrations (see also Table **2** of Supplementary Material).

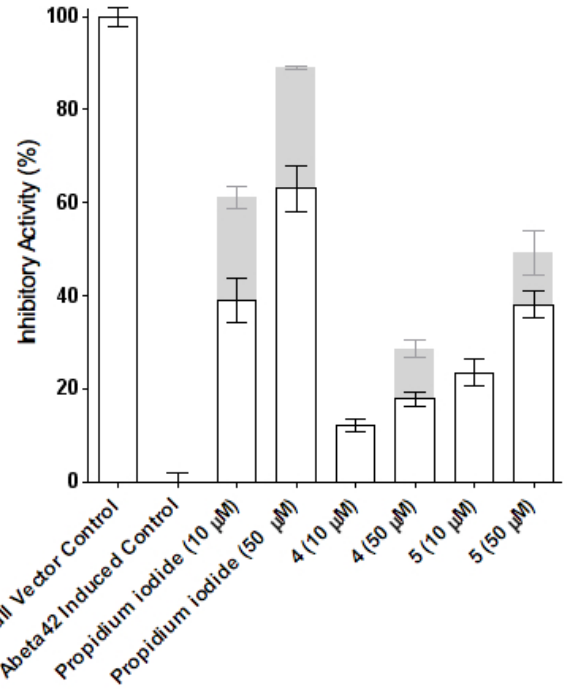

Fig. (5). Dosage of anti-aggregating activity monitored by Th-S staining of bacterial cells overexpressing Aβ42 peptide. Note that the grey section in each bar denotes the reported value of each inhibitor obtained by *in vitro* determinations [42,49].

Given the structural and tinctorial similarities between amyloid aggregates of different proteins, we thought that our methodology might be applied to other amyloidogenic proteins that form IBs when overexpressed in *E. coli* cells. Apart from Aβ42, another amyloidogenic protein, namely the tau protein, seems to play a pivotal role in the onset and progression of the neurodegenerative cascade of AD. This prompted us to assess the applicability of our methodology to the screening of the tau anti-aggregating effect of potential inhibitors in intact *E. coli* cells overexpressing tau protein. For this purpose, we selected tacrine and (±)-huprine Y, which had been found to be essentially inactive as Aβ42 aggregation inhibitors both *in vitro* and in *E. coli* cells, and compound (±)-**1**, which had been found to be active. As expected, when bacterial cells overexpressing tau protein were incubated in the presence of 10 μM of these selected compounds we found that tacrine and (±)-huprine Y were again inactive as anti-aggregating agents whereas heterodimer (±)-**1** inhibited tau protein aggregation in a very remarkable 67.6 ± 2.1% (Fig. **6**). These results support the applicability of this method for the screening of inhibitors of different disease-specific amyloidogenic proteins as well as the increasingly accepted notion that amyloidoses might be confronted with common therapeutic interventions [51].

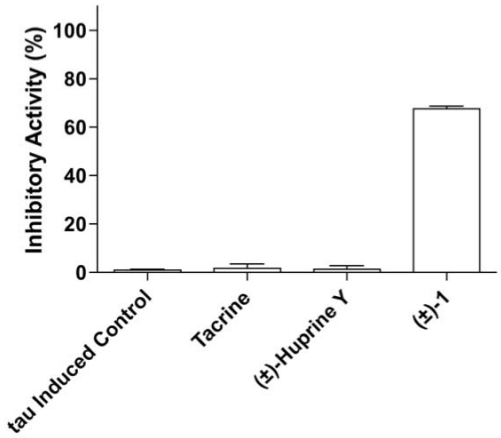

Fig. (6). Aggregation inhibitory activities of selected compounds monitored by Th-S staining of bacterial cells overexpressing tau protein. The graph shows the anti-aggregating effect of 10 μM of inhibitor added to bacterial culture overexpressing tau protein after overnight incubation.

The possibility of applying this method in automated technologies available for cell-based screening, like simple UV/Vis plate reader assays, might allow the *in vivo* analysis of large compound libraries at different stoichiometric ratios using this fast, simple, quantitative and cost-effective method.

4. CONCLUSION

Prokaryotic cells can provide a simple yet physiologically relevant model to study the mechanisms and extent of amyloid aggregation of peptides and proteins related to conformational diseases. Interestingly, bacterial systems can be easily implemented to study the effect of genetic mutations [52-55] and/or the effects of drugs [56] on the aggregation of amyloid-prone proteins. We present here a protocol in which the direct determination of the Th-S relative fluorescence of bacterial cells allows to monitor changes in the intracellular aggregation of amyloidogenic proteins, without the requirement for expensive equipment. The method can be potentially applied to track the effects of target compounds and sequential changes on the deposition of different amyloidogenic proteins. The ability of the method to recapitulate previous *in vitro* results regarding Aβ42 anti-aggregating activity suggests that it can become a standard able to replace non-physiological *in vitro* assays in the next future.

LIST OF ABBREVIATIONS

Aβ = β-Amyloid peptide

AD = Alzheimer's disease

APP = Amyloid precursor protein

CR = Congo Red

GFP = Green fluorescent protein

IBs = Inclusion bodies

IPTG = Isopropyl β-D-thiogalactopyranoside

PBS = Phosphate buffer saline

Th-S = Thioflavin-S

Th-T = Thioflavin-T

CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

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