Mining and engineering activity in catalytic amyloids

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Abstract

This chapter describes how to test different amyloid preparations for catalytic properties. We describe how to express, purify, prepare and test two types of pathological amyloid (tau and α-synuclein) and two functional amyloid proteins, namely CsgA from *Escherichia coli* and FapC from *Pseudomonas*. We therefore preface the methods section with an introduction to these two examples of functional amyloid and their remarkable structural and kinetic properties and high physical stability, which renders them very attractive for a range of nanotechnological designs, both for structural, medical and catalytic purposes. The simplicity and high surface exposure of the CsgA amyloid is particularly useful for the introduction of new functional properties and we therefore provide a computational protocol to graft active sites from an enzyme of interest into the amyloid structure. We hope that the methods described will inspire other researchers to explore the remarkable opportunities provided by bacterial functional amyloid in biotechnology.

Abbreviations

α-Syn α-Synuclein.
BCA Bicinchoninic acid.
FTIR Fourier transform infrared.
GdmCl Guanidinium chloride.
IPTG Isopropyl ß-D-1-thiogalactopyranoside.
MD Molecular dynamics.
MU 4-Methylumbelliferyl.

MUA	MU acetate.
MUB	MU butyrate.
MUO	MU oleate.
MUP	MU phosphate.
NC	Nitrocefin.
pLDDT	Per-residue confidence score.
pNP	4-Nitrophenyl.
pNPA	pNP acetate.
pNPB	pNP butyrate.
pNPP	pNP palmitate.
$pNPPO_4$	pNP phosphate.
QM/MM	Quantum mechanics/molecular mechanics.
RMSd	Root mean square deviation.
TCEP	Tris(2-carboxyethyl)phosphine.
VMD	Visual molecular dynamics.

1. Introduction

Although the term "protein amyloid" usually conjures up vaguely sinister associations with neurodegenerative diseases such as Alzheimer's and Parkinson's, it deserves a better press. Amyloid or cross-β structure is simply a structural arrangement involving a very efficient and robust way to self-assemble identical copies of a protein sequence. That is, individual β-strands are stacked on top of each other, held together by hydrogen bonds and creating a fibril whose long axis is perpendicular ("cross") to the β-strands. Individual fibrils formed by single strands have two surfaces but no buried core sequestered from the solvent (Fig. 1a). This can to some extent be remedied by associating two fibrils that bury one surface each, but that involves a quaternary arrangement between individual species which opens up for multiple different contacts and thus reduces the homogeneity of the amyloid species. However, if the individual units in this fibril form a β-hairpin (through the linking of two β-strands), it is possible to "control the narrative" much better by forming a buried core enclosed by the two strands (Fig. 1b). In this way, when the individual β-hairpins stack on top of each other, we obtain what corresponds to an infinitely elongated globular protein, stabilized both by hydrogen bonds between orthogonally stacked strands and by side-chain interactions (typically hydrophobic) within two strands in the same plane. At the same time, we obtain two extended surfaces on either side of the fibril which are (or can be) optimized for solvent exposure through appropriate polar and charged residues and — if necessary — modified for whatever activity we want them to possess. Such an example could

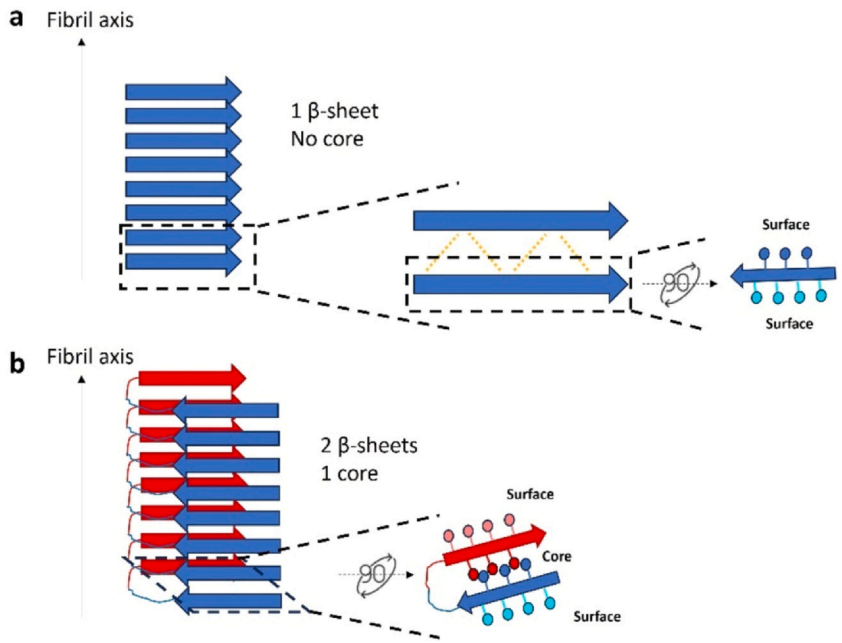

Fig. 1 ***Surface and core in amyloid.*** The figure shows the structural consequences of stacking (A) single peptide strands or (B) β-hairpins as cross-β amyloid. Orange stippled lines indicate backbone hydrogen bonding (for simplicity only shown in the zoom-in in panel A). Side chains alternately point up and down along the β-strand (shown in light and dark color). A single β-strand arrangement (panel A) leads to a single β-sheet with two surfaces, while a β-hairpin (panel B) allows a stabilizing core to form between the two strands, while leaving two surfaces that can be engineered for new properties, *e.g.* catalysis. This arrangement is found in functional amyloid such as the curli protein CsgA from *E. coli*.

include interactions with substrates to convert them into products. Obviously other structural stacking motifs can be formed besides the β-hairpin, but here we just consider the simplest case.

Such a β-hairpin arrangement is found in Nature among some members of the class of functional amyloid, that is amyloid produced by organisms for a specific purpose. Although functional amyloid is found in many different organisms, both high and low (though not, it seems, to any large extent in plants (Mohammad-Beigi et al., 2019)), it is particularly instructive to study them in bacteria, which have managed to integrate the amyloid state into their lifestyle choices in a very elegant way. The amyloid provides mechanical strength to the biofilm matrix in which the bacteria are embedded (Schwartz, Syed, Stephenson, Rickard, & Boles, 2012; Zeng et al., 2015), providing

protection against various kinds of predation including antibiotic attack. In the two most well-studied cases, that of curli in *Escherichia coli* and Fap in *Pseudomonas*, one major amyloid-forming protein (CsgA or FapC) is transported to the bacterial surface with the help of a whole team of ancillary proteins (see (Otzen and Riek, 2019; Peña-Díaz, Olsen, Wang, & Otzen, 2024; Sønderby, Najarzadeh, & Otzen, 2022a) for details). Once outside the cells, the protein is incorporated into preexisting fibrils which extend far from the outer membrane, entangling physically with neighboring cells to provide mechanical stability. The helper proteins include chaperoning proteins (in the curli system called csgC and csgE), transcriptional regulators (csgD), nucleators (csgB) and transporter (csgG and csgF). The proteins prevent aggregation in the periplasm, provide a channel through the outer membrane, and help the amyloid protein nucleate on the surface of the bacterium. In addition to this dedicated protein orchestra, the sequence of the main player (the amyloidogenic protein) has been optimized for fast and focused fibrillation. Both CsgA and FapC consist of multiple repeats of a relatively short (20 and 35 residues, respectively) sequence motif. This sequence organization has important consequences both for structure and self-assembly mechanisms. Put simply, repeats simplify both properties. In the case of CsgA, a combination of bioinformatic analyses, molecular dynamics (MD) simulations and cryoEM guidelines have revealed the repeat motif to form the simple β-hairpin motif illustrated in Fig. 1b, which in the amyloid structure is elongated as a long uniform cylinder (Sleutel, Pradhan, Volkov, & Remaut, 2023; Tian et al., 2015). The situation is a little more complicated for FapC where the longer repeat found in this class of proteins has been predicted to lead to a Greek key-like β-solenoid structure not found outside *Pseudomonas* (Mesdaghi, Price, Madine, & Rigden, 2023; Sønderby et al., 2022a) and thus a more convoluted surface than that of CsgA. Mechanistically the repeats reduce the complexity of the self-assembly process by favoring a minimalist nucleation-elongation model which leads to a steady growth of fibrils over time, compatible with the doubling times of the host bacteria (Meisl et al., 2022). This contrasts with pathological amyloid, whose aggregation mechanism tends to be dominated by secondary processes such as fragmentation and secondary nucleation, both of which lead to more explosive fibril growth. Removal of some or all of the repeats in FapC increases the tendency to fragmentation (Rasmussen et al., 2019) as well as decreasing fibril stability (Christensen, Nowak, Sønderby, Frank, & Otzen, 2020). This highlights the role of a minimal number of repeats in stabilizing both structure and assembly of functional amyloid.

Indeed, the extraordinary robustness of functional amyloid is a marked advantage for nanomaterial applications: FapC and CsgA fibrils only dissolve

at >60% formic acid (Dueholm et al., 2010, 2011) (α-synuclein (α-Syn) fibrils disintegrate below 0.1% FA (Christensen et al., 2020)), and neither extreme pH or temperature seem able to disintegrate them. Fibrillation can still occur at high molar concentrations of urea (Sønderby, Rasmussen, Frank, Skov Pedersen, & Otzen, 2022b). The only way to avoid amyloid is to target it at the early stage of fibrillation, where universal amyloid inhibitors such as polyphenols (Najarzadeh et al., 2019) or designed peptides targeting specific parts of the amyloid sequence (Sønderby et al., 2023) can block aggregation or divert the proteins to less stable amorphous aggregates. These aggregates are more easily dissolved in formic acid and protect the biofilm to a smaller extent, leading to lower biofilm yields (Najarzadeh et al., 2019) and lower antibiotic resistance (Stenvang et al., 2016).

The regular structure and high stability of the functional amyloid framework provides an excellent platform for engineering useful properties. As we have described in a recent review (Peña-Díaz et al., 2024), CsgA amyloid has been functionalized by different investigators to perform many different but mainly structural tasks. For example, attaching a C-terminal peptide extension or fusion protein with affinity for different materials promotes adherence to specific surfaces (Biofilm-Integrated Nanofiber Display or BIND (Nguyen, Botyanszki, Tay, & Joshi, 2014); Zhong et al., 2014). Attaching a mucin-binding protein improved the ability of *E. coli* to colonize the gastrointestinal tract of mice, allowing them to ultimately be applied for various intestinal disorders (Duraj-Thatte et al., 2019). In another twist, fusion with a trefoil protein led to an "aquaplastic" material that could not only be molded under water but also self-healed when damaged (Duraj-Thatte et al., 2021).

However, one aspect that remains to be explored further in functional amyloid is that of catalysis. The repetitive nature of the amyloid surface means that catalytic residues present in individual amyloid subunits will be displayed in a regular fashion, providing a highly active surface and a high binding capacity. Such a set-up allows for very efficient processing of substrate. For example, a polymer consisting of multiple scissile bonds could be cleaved repetitively as it diffuses across the amyloid surface, provided a significant proportion of the encounters are productive. As described in several other chapters in this volume, numerous different naturally occurring amyloids have some—albeit weak—catalytic activity, hydrolyzing a wide range of substrates. Jelinek and coworkers have pioneered the field with observations on the catalytic properties of amyloid

formed by the Aβ peptide (Arad, Baruch Leshem, Rapaport, & Jelinek, 2021), glucagon (Arad et al., 2022) and the PSMα3 peptide from *S. aureus* (Arad et al., 2023). The Wittung-Stafshede group has followed up on this by showing that α-syn fibrils not only catalyze hydrolytic processes *in vitro* with Michaelis-Menten type kinetics (Horvath and Wittung-Stafshede, 2023) but also seem to impact the metabolite population in cell extracts, although no specific reactions have been proposed at this stage (Horvath, Mohamed, Kumar, & Wittung-Stafshede, 2023). Korendovych and coworkers in turn have developed elegant peptide systems where amyloid formation is coordinated with metal binding, leading to a wide range of catalytic reactions depending on the metal ion involved (Rufo et al., 2014). In our view, the obvious next step is to graft existing active sites from known and well-characterized enzymes into the amyloid fold of functional amyloid such as CsgA and FapC. This has several advantages: besides the presence of a large number of active sites on a chemically and physically very robust structural platform, the amyloid surface is generally flat and therefore allows interactions with substrates such as large polymers that might find it difficult to engage with buried structures such as clefts or tunnels. CsgA is particularly suited because of the presence of two well-defined surfaces on either side of the core. FapC's more complex and convoluted surface provides some additional opportunities. Its predicted Greek key fold could be used to graft more complex catalytic configurations in different 3D arrangements. As evidence of this active surface, FapC has already been shown to be able to adsorb a range of different small metabolites with μM affinity, suggesting a role in maintaining a "sponge-like" binding buffer capacity which is useful in a complex environment with competition for nutrients (Seviour et al., 2015).

To the best of our knowledge, there have been no attempts to graft active sites into amyloid as yet, though enormous progress is being made in complementary approaches, such as the Baker lab's recent successes in designing enzymes around a given substrate using "constrained hallucination" or "inpainting" (Wang et al., 2022). We are only at the beginning of an exciting period of discovery where we need not only to chart the contours of the catalytic activity of existing amyloid but explore different strategies to engineer new properties into the amyloid structure. Accordingly, the present chapter describes how to tackle this as we will elaborate in the coming section.

2. General strategy to screen for activity *in vitro*

The current chapter provides a detailed and easy-to-implement workflow to purify and form amyloid fibrils and characterize their potential catalytic activity. As examples, we use different types of amyloids, namely two functional (CsgA and FapC) and two pathological, tau and α-Syn. We start by describing the expression and purification protocols of these four different amyloid proteins. The protocols have been extensively optimized to ensure the highest yields and purest monomeric protein possible with the simplest requirements in terms of equipment, reagents, and steps. Subsequently we go through the preparation of large amounts of homogenous amyloid fibrils prepared from their non-fibrillated precursors. As an additional twist, we describe how to generate multiple polymorphs of the same amyloid protein to study the impact of different structural arrangements. Having obtained these fibrils, we provide methods to validate the formation of amyloid fibrils. Thioflavin-T (Th-T) fluorescence is the standard approach, but since Th-T binding affinity can vary depending on the amyloid protein or the polymorph type, we also include alternative protocols to confirm the aggregation or the β-sheet content independent of Th-T fluorescence.

All this is a preamble to the main goal of the present work, namely, to provide a robust strategy to characterize fibrils' potential catalytic properties. Standard substrates for esterase-, phosphatase-, lipase-, lactamase- and anhydrase-like activities are described, along with protocols designed to maximize product formation and minimize (or at least compensate for) potential scattering background derived from the presence of fibrils. Fluorescent alternatives are provided to reduce the impact of this background. Importantly, this approach facilitates traditional Michaelis-Menten kinetic analysis and associated kinetic constants through recordings at different substrate concentrations. We also report procedures to characterize the catalytic capacity in greater detail. These include fibril sonication to evaluate the effect of the surface:volume ratio (affecting substrate accessibility and concentration of available active sites), the resistance of amyloid to extreme conditions and their effects on catalytic activity.

While this chapter aims to provide the user with the basic tools for performing catalytic analysis and screening of amyloid fibrils, we emphasize that procedures should be adapted as required for the particular amyloid or activity studied.

3. Materials and equipment

3.1 Protein expression and purification

Table 1 List of reagents and consumables used for protein expression and purification.

Reagent	Supplier	Reference
Tryptone	Sigma	61044
Yeast extract	Sigma	70161
NaCl	Sigma	S9888
Agar	Sigma	05039
Ampicillin sodium salt	Sigma	A9518
Kanamycin sulfate	Sigma	60615
Gentamycin sulfate	Sigma	G1264
Guanidinium chloride (GdmCl)	ThermoScientific	A13543
Tris BASE	Fisher	93352
HCl	ThermoScientific	124620010
Imidazole	Sigma	56750
IPTG (Isopropyl ß-D-1-thiogalacto-pyranoside)	Sigma	I5502
EDTA	Sigma	E4884
$MgSO_4$ + $7H_2O$	Sigma	230391
$(NH_4)_2SO_4$	VWR Chemical	855001.290
KH_2PO_4	PanReac	A1043,1000
Na_2HPO_4 anhydrous	VWR Chemical	28029.292
Glycerol	Sigma	G7757
D-Glucose	Sigma	G7021
D-Lactose	Sigma	61345
Peptone	Sigma	70169
D-Sucrose	Fisher	BP220-1
TCEP	Sigma	C4706
Ni^{2+} NTA agarose beads	MC-LAB	NINTA-500

(*continued*)

Table 1 List of reagents and consumables used for protein expression and purification. (*cont'd*)

Reagent	Supplier	Reference
HiTrap Q HP anion exchange column	Cytiva	17115401
HiTrap SP HP cation exchange column	Cytiva	17115201
NaOH	ChemSOLUTE	1375.1000
Acetic acid	Sigma	695092
Spectra/Por® 3 Dialysis Membranes, MWCO 3500	Spectrum® Laboratories (VWR Chemical)	28170-166
EDTA-free Protease Inhibitor Cocktail	Roche (Sigma)	05892791001

3.2 Protein aggregation, validation, and isolation

Table 2 List of reagents and consumables used for protein aggregation and validation.

Reagent	Supplier	Reference
KCl	Sigma	P3911
Tris BASE	Fisher	93352
NaCl	Sigma	S9888
TCEP (tris(2-carboxyethyl)phosphine)	Sigma	C4706
Na_2HPO_4 anhydrous	VWR Chemical	28029.292
KH_2PO_4	PanReac	A1043,1000
Thioflavin-T	Sigma	T3516
Heparin sulfate	Sigma	H4784
Spectra/Por® 3 dialysis membranes, MWCO 3500	Spectrum® Laboratories (VWR Chemical)	28170-166
PD MidiTrap G-25	Cytiva	28918008
Glass beads 3 mm	Sigma	1040150500
Half area 96-well Black, clear bottom polystyrene microplates	Corning	3881
96-well black, clear bottom polystyrene microplates	Corning	3631
BCA kit reducing conditions	ThermoFisher	23250

3.3 Enzymatic screening and activity characterization

Table 3 List of reagents used for catalytic assays.

Reagent	Supplier	Reference
Tris BASE	Fisher	93352
4-Nitrophenyl acetate (pNPA)	Sigma	N8130
4-Nitrophenyl phosphate ($pNPPO_4$)	Sigma	N3254
4-Nitrophenyl butyrate (pNPB)	Sigma	N9876
4-Nitrophenyl palmitate (pNPP)	Sigma	N2752
Nitrocefin (NC)	Sigma	484400
4-Methylumbelliferyl acetate (MUA)	Sigma	M0883
4-Methylumbelliferyl phosphate (MUP)	Sigma	M8883
4-Methylumbelliferyl oleate (MUO)	Sigma	75164
4-Methylumbelliferyl butyrate (MUB)	Sigma	19362
Carbonic anhydrase	Sigma	C3934
Phenol red	Sigma	114529
CO_2	Linde Gas	UN1013
DMSO	Fisher	10122140
Acetonitrile	Fisher	11373230
96-well black, clear bottom polystyrene microplates	Corning	3631

4. Protein expression and purification

Protein expression and its subsequently purification in acceptable levels can become a major bottleneck if a multitude of assays has to be run with the amyloid of interest. Accordingly, designing and optimizing protocols that lead to the highest yield possible is crucial. In the following sections we will detail optimized strategies to purify various amyloid proteins in sufficient amounts as to perform the different catalytic assays here described. A list of reagents to use is provided in Table 1.

4.1 Materials, media, and buffers preparation

4.1.1 Bacterial cultures and antibiotics

1. LB media:
   - Add ~600 mL of MQ water.
   - While stirring with a magnetic bar, add 16 g Tryptone followed by 10 g yeast extract and 5 g NaCl.
   - Once dissolved, add MQ water to make 1 L
   - Autoclave.
2. LB-agar media:
   - Add 200 mL of LB media into a 200 mL bottle.
   - Add 3 g of agar.
   - Autoclave.
3. 2xYT media
   - Add ~600 mL of MQ water.
   - While stirring, add 10 g Tryptone followed by 5 g yeast extract and 10 g NaCl.
   - Once dissolved, add MQ water to make 1 L
   - Autoclave.
4. Magnesium sulphate ($MgSO_4$) 1 M
   - Add ~100 mL of MQ water.
   - Add 24.64 g $MgSO_4 + 7\ H_2O$.
   - Dissolve using a magnetic stirrer.
   - Autoclave.
5. NPS solution 20X:
   - Add ~1800 mL MQ Water
   - While stirring add 132 g (NH4)2SO4, 272 g KH2PO4 and 284 g Na2HPO4 anhydrous.
   - Once dissolved, add MQ water to make 2000 mL.
   - Autoclave
6. 5052 solution 50X:
   - Add ~700 mL MQ Water
   - While stirring and gently heating, add 250 g of glycerol 100%, 27 g of glucose monohydrate and 284 g lactose monohydrate.
   - Once dissolved, add MQ water to make 1000 mL.
   - Autoclave
7. Autoinduction media:
   - Add ~3000 mL MQ Water
   - While stirring, add 8 g of yeast extract and 40 g of peptone.

- Add 8 mL of MgSO4 1 M, 80 mL of 5052 solution 50X and 200 mL of NPS solution 20X.
- Once dissolved, add MQ water to make 4000 mL.
- Autoclave

8. Ampicillin 100 mg/mL
   - Add 1 g of ampicillin sodium salt.
   - Add 7.5 mL of MQ water.
   - Mix using a vortexer.
   - Add MQ water to make 10 mL.
   - Filter through a 0.22 μm filter.
9. Kanamycin 50 mg/mL
   - Add 2 g of Kanamycin sulfate salt.
   - Add 20 mL of MQ water.
   - Mix using a vortexer.
   - Add MQ water to make 40 mL.
   - Filter through a 0.22 μm filter.
10. Gentamycin 20 mg/mL
    - Add 1 g of Gentamycin sulfate.
    - Add 40 mL of MQ water.
    - Mix using a vortexer.
    - Add MQ water to make 50 mL.
    - Filter through a 0.22 μm filter.
11. IPTG 1 M
    - Add 4.762 g of IPTG.
    - Add 15 mL of MQ water.
    - Mix using a vortexer.
    - Add MQ water to make 20 mL.
    - Filter through a 0.22 μm filter.

4.1.2 Protein purification stocks

1. Tris/HCl 1 M:
   - Add ~750 mL of MQ water.
   - While stirring, add 121.14 g Tris BASE.
   - Once mixed, adjust the pH as corresponding using HCl
   - Add MQ water to make 1 L
   - Filter through a 0.22 μm filter.
2. NaCl 5 M:
   - Add ~750 mL of MQ water.
   - While stirring and heating, add 292.2 g NaCl.

- Once mixed, add MQ water to make 1 L
- Filter through a 0.22 μm filter.

3. Guanidinium chloride (GdmCl) 7.5 M:
   - Add ~400 mL of MQ water.
   - While stirring and heating, progressively add small amounts of GdmCl until have 726.55 g.
   - Once mixed, add MQ water to make 1 L.
4. Imidazole 1 M:
   - Add ~700 mL of MQ water.
   - While stirring, add 68,08 g of imidazole.
   - Once mixed, add MQ water to make 1 L
5. EDTA 0.5 M pH 8.0:
   - Add ~800 mL of MQ water.
   - While stirring, add 186.16 g of disodium EDTA dihydrate.
   - While mixing, adjust the pH to 8.0 by adding NaOH 6 M.
6. Osmotic shock buffer:
   - Add ~400 mL of MQ water.
   - While stirring, add 24 mL Tris/HCl 1 M, 320 g sucrose and 3.2 mL EDTA 0.5 M.
   - Adjust the pH to 7.2.
   - Once mixed, add MQ water to make 800 mL
7. TCEP 1 M:
   - Add 2.8665 g TCEP.
   - Add 7.5 mL of MQ water.
   - While mixing, using a vortexer
   - Once mixed, add MQ water to make 10 mL.
   - Filter through a 0.22 μm filter.

4.2 CsgA purification

CsgA protein is the main component of the curli system and one of the most studied functional amyloids. While different curli systems have been discovered in the *Enterobacter* family and exhibit clear differences, they all share common features that can be exemplified with the *E. coli* curli protein. While CsgA aggregation in vivo is extremely regulated by a complex system that is described above, CsgA readily forms amyloid fibrils in absence of these components *in vitro* by adopting a β-hairpin motif. The large aggregation propensity of CsgA complicates the purification process as its overexpression leads to the presence of fibrils. Therefore we have developed

and optimized an expression and purification protocol, described below, that exploits a C-terminal His tag to perform Ni^{2+}-based affinity chromatography under highly denaturing conditions.

4.2.1 Cell plating

1. Prepare LB-agar media by adding 3 g agar in 200 mL of LB (10 g tryptone, 5 g yeast extract and 10 g NaCl/1 L) and autoclave.
2. Under sterile conditions add 50 μL of 100 mg/mL Ampicillin into a 50 mL falcon and immediately fill with 50 mL of solubilized warm (but not hot) LB-agar. This gives a final Ampicillin concentration of 100 μg/mL.
3. Carefully mix the antibiotic in the LB-agar by simply inverting the tube 4–6 times and rapidly aliquot 10 mL in 60 mm Petri dish plates.
4. Let the plates cool down under sterile conditions for 5–10 min in a laminar flow bench without the lid to avoid condensation.
5. Pick a CsgA-expressing *E. coli* colony obtained from a glycerol stock (BL21 DE3, SlyD/CsgA WT) with a sterile loop and spread it onto the Ampicillin containing plates.
6. Let it dry for 5–10 min and incubate at 37 °C overnight with the LB-agar layer placed on top.
   Note 1: We warmly recommend to use a BL21-DE3 slyD knockout strain. The protein SlyD is naturally upregulated during CsgA overexpression and will co-purify with CsgA as it also binds strongly to Ni^{2+}-NTA beads.
   Note 2: Prepare 2 plates/1 L of culture.

4.2.2 Overnight culture

1. Under sterile conditions add 10 μL of 100 mg/mL Ampicillin into a 50 mL falcon and immediately fill with 10 mL of solubilized 2xYT media (16 g Tryptone, 10 g yeast extract and 5 g NaCl/1 L)/1 L of culture. Final concentration of Ampicillin is 50 μg/mL.
2. Pick one colony with a sterile loop and inoculate the O/N culture media.
3. Finally, incubate at 37 °C and 140 rpm overnight.
   Note 1: Prepare the 2xYT media (16 g Tryptone, 10 g yeast extract and 5 g NaCl/1 L) the day before the culture is started to reach a proper temperature.
   Note 2: The same picked colony may be used to inoculate 2 falcon tubes.
   Note 3: Instead of 50 mL falcon tubes, fresh LB-agar plates could be used as O/N culture. In this case, prepare 2 plates/1 L of culture.
   Note 4: It is important to leave partially open the falcon tubes (secured with adhesive tape) to maximize oxygenation. Also, the falcon tubes should be placed at an angle using a rack.

4.2.3 Protein expression

1. Add 1 mL of 100 mg/mL Ampicillin (final concentration 50 μg/mL) to the 2xYT media.
2. Recover the ON culture into a single falcon and mix it.
3. Add equivalent volumes to the culture media and close it with autoclaved foam pieces.
4. Incubate the flasks at 37 °C and 140 rpm.
5. Measure OD at 600 nm every hour.
6. Once OD has reached 1.7 (usually 3–4 h), induce protein expression by adding 100–150 μL of IPTG 1 M.
7. Incubate for an additional 1 h at 37 °C at 140 rpm.
8. Harvest the cells by centrifugation at 4.000 rpm for 15 min in a centrifuge.
9. Transfer the pellets to 50 mL tubes by resuspending in PBS 1X.
10. Centrifuge the cells again at 4000 rpm for 15 min
11. Finally, save and store the pellets at −80 °C.

Note 1: Keep 2–4 mL of 2xYT media to use as a blank or dilution media before inoculating the bacteria.

Note 2: To measure OD over 0.8–1, the culture should be diluted (a 1:2 dilution should suffice).

Note 3: Instead of resuspending in PBS 1X and freezing, the pellets may be resuspended in 100 mL/L of culture of 7.5 M GdmCl, 50 mM Tris/HCl pH 7.4. The resuspended pellets should be incubated at 4 °C overnight with gentle stirring. In case the pellets are frozen, this step should be performed the day before to the purification.

Note 4: The pellets can be stored for up to a week.

4.2.4 Protein purification

1. If not performed immediately after recovering the pellets, thaw and then resuspend the pellets in 100 mL/L of culture of 7.5 M GdmCl, 50 mM Tris/HCl pH 7.4.
2. Incubate the resuspension at 4 °C overnight with gentle stirring.
3. Centrifuge the incubated sample in 50 mL falcon tubes at 12,000 g for 20 min
4. Meanwhile, put 2–2.5 mL of Ni-NTA beads per liter of culture in a 50 mL tube and fill it with MQ water.
5. Mix and centrifuge for 1 min at 1000 rpm with slow deceleration to avoid pellet resuspension.

6. Carefully discard the supernatant from the Ni-NTA beads, bearing in mind that the pellet is extremely soft and easily resuspended. Repeat this washing step at least 3 times.
7. Recover the GdmCl-containing supernatant from the cell lysate and mix it for 1 h with the cleaned Ni-NTA beads by gentle stirring.
8. Afterwards, transfer and centrifuge the mix in two 50 mL tubes for 1 min at 1000 rpm with slow deceleration.
9. Once the complete sample is centrifuged, wash the beads twice with 40–50 mL of 7.5 M GdmCl, 50 mM Tris/HCl pH 7.4 and centrifuge for 1 min at 1000 rpm with slow deceleration.
10. Then, wash twice again with 7.5 M GdmCl, 50 mM Tris/HCl pH 7.4, 20 mM imidazole to remove contaminants and centrifuge for 1 min at 1000 rpm with slow deceleration.
11. Transfer the beads by using 30 mL of 7.5 M GdmCl, 50 mM Tris/HCl pH 7.4, 20 mM imidazole into 2 cleaned columns. Fill the columns with the same buffer and let it elute.
12. Finally, carefully add 15 mL of 7.5 M GdmCl, 50 mM Tris/HCl pH 7.4, 500 mM imidazole to elute the protein.
13. Recover the eluted samples in 1.5 mL fractions and measure the concentration using Nanodrop, setting the molecular weight to 15049.15 Da and ε_{280} to 11460 $M^{-1}cm^{-1}$.
14. Mix the samples containing more than 0.5 mg/mL and measure the concentration again.
15. The sample should now be aliquoted in 0.5–1 mL portions and frozen at −80 °C until use.

    Note 1: Do not add more than 45 mL in each 50 mL tube for centrifugation.

    Note 2: Due to the high centrifugation speed, the tubes should be carefully equilibrated with weight differences < 0.1 g.

    Note 3: Recover and regenerate Ni^{2+}-NTA beads. Fill the columns with washing solution (NaOH 0.5 M). After eluting, repeat the process with 100 mM EDTA solution to remove the remaining Ni^{2+}; as Ni^{2+} is toxic, use different bottles for recovering the elution. Then, fill the columns with Ni (II) sulphate solution 2.6% and elute until Ni^{2+} starts eluting. Close the columns (both bottom and top) and incubate with horizontal shaking for 5 min to maximize Ni^{2+} binding. Elute the remaining $NiSO_4$ solution and fill the column with regeneration buffer (0.1 M acetic acid). Clean and store the beads in 30% ethanol.

4.3 FapC purification

Like CsgA, FapC protein is another but less explored example of functional bacterial amyloids from *Pseudomonas* strains. Similar to CsgA, the sequence of FapC contains a variable number of repeats that seem to play a key role in the aggregation process. Moreover, *in vivo* aggregation is also tightly controlled for multiple proteins that act as chaperones (fapD), fibril modulators (fapA), nucleators (fapB) and transporter (fapF, fapD and fapE). Although FapC presents a slightly lower aggregation tendency than CsgA, its overexpression also leads to the presence of fibrils. Therefore, we describe an optimized expression and purification protocol using the same His-tag extension and Ni-NTA based denaturing chromatography which we described for CsgA.

4.3.1 Cell plating, overnight culture and protein expression and purification

Repeat 4.2.1–4.2.4 using 50 μg/mL Kanamycin (from a 50 mg/mL stock) instead of ampicillin and using a FapC glycerol stock (BL21 DE3/FapC WT) (without signal sequence).

4.4 Tau2N4R purification

Tau is an intrinsically disordered protein associated with microtubule stabilization and axonal transport on healthy conditions. However, it is also implicated in the onset and progression of neurodegenerative diseases like Alzheimer's by forming amyloid fibrils upon hyperphosphorylation. The sequence composition of the protein allows to distinguish 4 main regions: (i) the N-terminal domain with two amino acid inserts referred as N1 and N2; (ii) a proline-rich segment; (iii) the microtubule binding domain, which contains 3 or 4 imperfect repeats (R1, R2, R3 and R4) and presents two highly aggregation prone hexapeptides at R1 and R2; and (iv) the C-terminal domain. Note that tau is expressed as different isoforms that vary depending on the presence of the R2 repeat or as well as the N-terminal inserts (N1 and N2). The purification of tau2N4R, considered the wildtype and most frequent version in aggregates, usually leads to low yields of protein. Nevertheless, in the following section we describe an optimized expression and purification protocols that leads to amounts sufficient for screenings of modestly sized chemical libraries. We exploit tau's close-to-physiological-pH pI (8.24) to perform a 2-step ion exchange chromatography.

4.4.1 Overnight culture

Repeat 4.2.1 using 100 μg/mL Ampicillin and 20 μg/mL Gentamycin (from 100 and 20 mg/mL stocks, respectively) instead of just 100 μg/mL Ampicillin and a tau2N4R glycerol stock (pET16b/tau2N4R).

4.4.2 Protein expression

1. Prepare 4 L of autoinduction media by mixing 20 g of yeast extract, 40 g peptone, 8 mL of $MgSO_4$ 1 M, 80 mL of 5052 solution 50X (250 g glycerol 100%, 27.5 g glucose monohydrate, and 105 g lactose monohydrate/1 L) and 200 mL of NPS solution 20X (132 g $(NH_4)_2SO_4$, 272 g KH_2PO_4, 284 g Na_2HPO_4 anhydrous/2 L).
2. Under sterile conditions, resuspend the bacteria from the overnight plate in 4.4.1 in 40 mL of the autoinduction media using a cell spreader.
3. Transfer the resuspended bacteria to an Erlenmeyer flask containing autoinduction media with 100 μg/mL Ampicillin and 20 μg/mL Gentamycin.
4. Incubate the flasks at 30 °C with shaking (250 rpm).
5. Measure OD at 600 nm every 1–2 h.
6. Once OD has reached 1.5-1.8 (c. ~6–7 h), harvest the cells by centrifugation at 4.000 rpm for 20 min
7. Transfer the pellet to 50 mL tubes using 20–25 mL Tris 20 mM, pH 7.4 per pellet. Save and store the pellets at −80 °C.
   Note 1: Keep 2–4 mL of autoinduction media to use as a blank or dilution media before inoculating the bacteria.
   Note 2: To measure OD over 0.8–1, the culture should be diluted (a 1:2 dilution should suffice).
   Note 3: The pellets can be stored for up to a week.

4.4.3 Sonication and centrifugation

1. Prior to sonication, dilute the pellets in column buffer (20 mM Tris, 1 mM TCEP pH 7.4) up to max 35 mL and add a protease inhibitor tablet to each tube to prevent proteolytic degradation.
2. Sonicate the pellets with a rod sonicator on ice for around 40 s ON and 20 s OFF at least 7 times.
3. Centrifuge the sonicated cells at 12.500 rpm and 4 °C for 10 min
4. Finally, save and store the supernatant until the chromatography step below
   Note 1: Check that the samples are not warm after sonication.
   Note 2: To avoid extreme heating, rotate the samples. Keep the samples always on ice and only take them out to be sonicated immediately before use.

4.4.4 Cation exchange chromatography

1. Prepare 250 mL Buffer A (20 mM Tris, 1 mM TCEP, 1 mM EDTA, pH 6.25) and 200 mL Buffer B (20 mM Tris, 1 mM TCEP, 1 M NaCl, pH 6.25).

2. Adjust pH after addition of TCEP.
3. Filter both buffers and degas before using.
4. Put pumps A1 and B1 in MQ.
5. Set alarm pressure to 0.5 MPa, system flow to 0.5 mL/min with pre-column pressure and monitor absorption at 215 nm.
6. Select downflow.
7. Attach the columns to the system.
8. Perform a pump wash of both pumps.
9. Run c. 30 mL water.
10. After 5 column volumes set system flow to 1 mL/min
11. Pause and introduce the pumps into the corresponding buffers.
12. Perform again a pump wash of both pumps.
13. Set system flow to 2 mL/min and inject 100% of buffer B to clean the columns
14. Once the conductivity reaches plateau (c. 15–20 mL of buffer B) run 40 mL of buffer A
15. When the conductivity is at baseline (at least 15 mL at baseline), the supernatant can be loaded.
16. Transfer pump A to supernatant carefully and start again.
17. After injecting the supernatant, transfer pump A to buffer A.
18. Load 40 mL of buffer A until baseline.
19. Wash with 40 mL of 10% buffer B.
20. Set gradient target to 50% of buffer B during 40 min with a system flow of 1 mL/min.
21. Fractionate the eluted samples in 1.3 mL aliquots.
22. Finally, set to 100% of buffer B and run 40 mL to clean the columns and stop fractionation.
23. Save the fractions and run an SDS-PAGE to select the aliquots containing the protein.

    Note 1: TCEP significantly acidifies the media.

    Note 2: Two pre-packaged cationic columns should be used to purify for more than 2 L of medium.

    Note 3: Let buffer drip into the column before putting on the lid or connecting to the system to avoid air on the column.

    Note 4: When equilibrating the columns, run at least 2 column volumes at the plateau/baseline.

    Note 5: Lift the sample containing tube a little to remove bubbles beneath it when injecting.

Note 6: After cleaning with 100% buffer B, clean and recover the columns by cleaning first with 40 mL of MQ water and then with 40 mL of EtOH 20%. Remember to perform a pump wash when changing the buffer.

4.4.5 Anion exchange chromatography

1. Prepare 350 mL Buffer A (20 mM Tris, 1 mM TCEP, 1 mM EDTA, pH 9.0) and 200 mL Buffer B (20 mM Tris, 1 mM TCEP, 1 M NaCl, pH 9.0).
2. Adjust pH after the addition of TCEP.
3. Filter both buffers and degas before using.
4. Pool fractions containing protein in 100 mL of Buffer A (20 mM Tris, 1 mM TCEP, 1 mM EDTA, pH 9.0)
5. Put pumps A1 and B1 in MQ.
6. Set alarm pressure to 0.5 MPa, system flow to 0.5 mL/min with pre-column pressure and monitor absorption at 215 nm.
7. Select downflow.
8. Attach the columns to the system.
9. Perform a pump wash of both pumps.
10. Run c. 30 mL water.
11. After 5 column volumes set system flow to 1 mL/min
12. Pause and introduce the pumps into the corresponding buffers.
13. Perform again a pump wash of both pumps.
14. Set system flow to 2 mL/min and inject 100% of buffer B to clean the column.
15. Once the conductivity reaches plateau (c. 15–20 mL of buffer B) run 40 mL of buffer A
16. When the conductivity has reached baseline levels and remained at least 15 mL at baseline, the supernatant can be loaded.
17. Transfer pump A to supernatant carefully and start again.
18. After injecting the supernatant, transfer pump A to buffer A.
19. Load 40 mL of buffer A until baseline.
20. Wash with 40 mL of 10% buffer B.
21. Set gradient target to 50% of buffer B during 40 min with a system flow of 1 mL/min.
22. Fractionate the eluted samples in 1 mL aliquots.
23. Finally, set to 100% of buffer B and run 40 mL to clean the columns and stop fractionation.

24. Save the fractions and run an SDS-PAGE to select the aliquots containing the protein.
    Note 1: TCEP significantly acidifies the media.
    Note 2: It is extremely important to increase the pH of the samples from 6.25 to 9.0 so the protein may interact with the resin, which has an opposite charge to the previous step.
    Note 3: Let buffer drip into the column before putting on the lid or connecting to the system to avoid air on the column.
    Note 4: When equilibrating the columns, run at least 2 column volumes at the plateau/baseline.
    Note 5: Lift the sample containing tube a little to remove bubbles beneath it when injecting.
    Note 6: After cleaning with 100% buffer B, clean and recover the columns by cleaning first with 40 mL of MQ water and then with 40 mL of EtOH 20%. Remember to perform a pump wash when changing the buffer.

4.5 α-Syn purification

α-Syn is a 140 aa intrinsically disordered protein whose aggregation is associated with the onset and progression of Parkinson's disease (PD). The sequence of this protein is divided into 3 main regions: (i) the N-terminal domain, which contains most of the genetic mutations associated with familial cases of PD and most of the imperfect repeats that drive protein:lipids interactions; (ii) the NAC domain, a highly hydrophobic segment that leads and stabilizes β-sheets interactions; and (iii) the C-terminal domain, which contains a large number of acidic residues that provide a significant negative charge and acidic character (its pI is 4.67). In the following section we will describe a purification process that takes advantages of this highly anionic character for isolating and purifying large amounts of α-Syn.

4.5.1 Overnight culture

Repeat 4.2.1 using α-Syn glycerol stock (BL21/DE3, T1, α-Syn WT).

4.5.2 Protein expression

Repeat 4.4.2 using only 100 μg/mL Ampicillin as antibiotic.

4.5.3 Cell lysis and protein isolation

1. Prepare 100 mL of MQ water/L of culture in a bottle and leave at 4 °C (or ice) to cool down.

2. Prepare the osmotic shock buffer (24 mL Tris/HCl 1 M, 320 g sucrose and 3.2 mL EDTA 0.5 M pH 7.2/1 L).
3. Without thawing the cells, add osmotic buffer to the falcon tubes and, using a spatula, transfer to 500 mL centrifugal tubes.
4. Recover the leftovers by adding more osmotic buffer to the tubes.
5. Quickly add the rest of the osmotic buffer into the tubes and equilibrate them.
6. Centrifuge at 7000 g for 30 min.
7. Carefully discard the supernatant.
8. Immediately add cold water (4 °C) to the pellets and transfer them into centrifuge tubes without resuspending.
9. Add 40 μL of saturated $MgSO_4$/100 mL of sample to each tube and gently mix for <3 min.
10. Centrifuge at 9000 g for 30 min.
11. Collect the supernatant and discard the pellet.
12. Using a pH-meter, acidify the supernatant to pH 3.5 adding 3-4 drops of HCl 6 M. If needed, adjust with HCl 1 M.
13. Then, centrifuge at 9000 g for 20 min and recover the supernatant.
14. Finally, adjust the pH to 7.5 and freeze the protein at −80 °C in 200 mL tubes.

    Note 1: It is important to *not* resuspend the cells when recovering with the osmotic shock buffer to avoid lysis and protein losses.

    Note 2: After the centrifugation with the osmotic shock buffer the pellets are extremely labile, so this step should be done cautiously to avoid losing cells.

    Note 3: Acidification is an extremely aggressive step of purification that might damage the protein of interest, so it should be done as quickly as possible.

    Note 4: If purified the same day, the freezing step may be avoided.

4.5.4 Protein purification

1. Prepare, filter, and degasify the buffers needed for the chromatography Buffer A (20 mM Tris/HCl pH 7.4) and Buffer B (20 mM Tris/HCl pH 7.4, NaCl 1 M).
2. Put pumps A1 and B1 in MQ.
3. Set alarm pressure to 0.5 MPa, system flow to 0.5 mL/min with pre-column pressure and monitor absorption at 215 nm.
4. Select downflow.
5. Attach the Q-sepharose columns to the system.

6. Perform a pump wash of both pumps.
7. Run c. 30 mL water.
8. After 5 column volumes, set system flow to 1 mL/min
9. Pause and introduce the pumps into the corresponding buffers.
10. Perform again a pump wash of both pumps.
11. Set system flow to 2 mL/min and inject 100% of buffer B to clean the column.
12. Once the conductivity reaches plateau (c. 15-20 mL of buffer B) run 40 mL of buffer A
13. When the conductivity is at baseline (at least 15 mL at baseline), the supernatant can be loaded.
14. Filter the protein sample with a 0.45 μm filter before using.
15. Transfer pump A to supernatant carefully and start again.
16. Inject the equivalent volume to up to 4 L of culture into the columns with a flow rate of 0.75 mL/min.
17. Wash with 100 mL of buffer A to reach the baseline.
18. Wash with 100 mL 10% of buffer B to remove contaminants
19. Set gradient target to 50% of buffer B and inject 120 mL with a system flow of 3 mL/min.
20. Collect fractions of 8–10 mL.
21. Finally, set to 100% of buffer B and run 40 mL to clean the columns and stop fractionation.
22. Save the fractions and run an SDS-PAGE to select the aliquots containing the protein.
23. Dialyze the protein against MQ water.
24. Finally, calculate protein concentration in a Nanodrop setting molecular weight to 14,460 Da and ε to 5960 $M^{-1}cm^{-1}$.
25. Lyophilize in 15 mL tubes for, at least, 48 h.

Note 1: Let buffer drip into the column before putting on the lid or connecting to the system to avoid air on the column.

Note 2: When equilibrating the columns, run at least 2 column volumes at the plateau/baseline.

Note 3: Lift the sample containing tube a little to remove bubbles beneath it when injecting.

Note 4: After cleaning with 100% buffer B, clean and recover the columns by cleaning first with 40 mL of MQ water and then with 40 mL of EtOH 20%. Remember to perform a pump wash when changing the buffer.

Note 5: Dialysis requires 2–3 buffer exchanges of at least 3–4 h (one of them overnight).

5. Protein aggregation protocols

Protein aggregation and, in particular, amyloid formation is a complex process that strongly depends on protein composition and environmental factors. Traditionally, amyloid aggregation has been envisioned as a 'simple' process in which a misfolded protein transition from its monomeric state into a fibrillar conformation upon the formation of intermediates structures as oligomers and protofibrils. In reality this process is extremely complex and heterogenous and requires significant optimization. Some proteins, such as functional amyloids, are capable of forming an homogenous sample of fibrils in short periods of time and under almost any condition. Other proteins like pathological amyloids are characterized by slower aggregation (though ultimately very rapid due to secondary processes) that might require the presence of cofactors and could lead to a severely heterogeneous fibril content. In the following sections we will describe a set of optimized protocols to follow up the aggregation of different types of amyloids (Fig. 2), either pathological or functional, but also to scale up the process to obtain sufficient amounts for catalytic assays. A list of reagents for these protocols is provided in Table 2.

5.1 Materials, media, and buffers preparation

5.1.1 Buffer stocks for aggregation

1. 1 M Tris/HCl pH 7.4

- Add 121.14 g Tris BASE
- Add MQ water to make 750 mL
- Mix using a magnetic stirring.
- Adjust pH using HCl to pH 7.4
- MQ water to make 1000 mL
- Filter through a 0.22 μm membrane

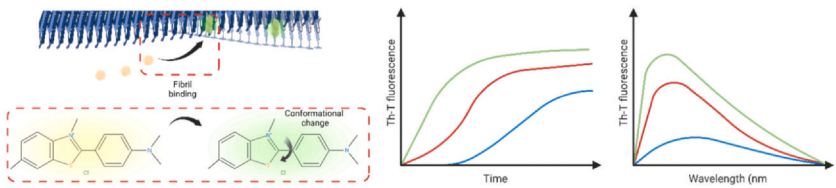

Fig. 2 Thioflavin-T and amyloid fibrils. Schematic representation of the conformational changes of Th-T upon fibril interaction and the resultant kinetics and final point measurements. Kinetic analysis provides important insight into the propensity to form amyloids. Shorter or non-existent lag phase, as well as higher signals (green) suggest indicate a higher amyloid propensity compared to slower reactions and less intense signal (blue).

2. TCEP 1 M:
   - Add 2.8665 g TCEP.
   - Add 7.5 mL of MQ water.
   - Mix using a vortexer
   - Once mixed, add MQ water to make 10 mL.
   - Filter through a 0.22 μm filter.
3. PBS 10X:
   - Add 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4 and 2.4 g KH_2PO_4.
   - Add 500 mL of MQ water.
   - Mix using a magnetic stirrer.
   - Adjust the pH to 7.4.
   - Add MQ water to make 1 L.
   - Autoclave.
4. KCl 1 M:
   - Add 74.55 g of KCl
   - Add 500 mL of MQ water.
   - Mix using a magnetic stirrer.
   - Once mixed, add MQ water to make 1 L.
   - Filter through a 0.22 μm filter.

5.1.2 Reporters and cofactor stocks

1. Thioflavin-T (Th-T) 500 μM:
   - Add 3.19 mg Th-T
   - Add 12 mL MQ water.
   - Mix using a vortexer.
   - Add MQ water to make 20 mL
   - Filter through a 0.22 μm membrane
   - Make aliquots and keep at −20 °C until use.
2. Heparin 2 mg/mL
   - Add 3 mg heparin.
   - Add 750 μL MQ water.
   - Resuspend carefully.
   - Make aliquots and keep at −20 °C until use.

5.2 CsgA aggregation

CsgA fibrillation *in vivo* is strongly controlled by the expression of the different components of curli system, allowing the formation of the extracellular matrix and biofilm. The lack of these components *in vitro*, however, leads to a fast aggregation process under multiple conditions, resulting in complete kinetics

within hours. In the following section we will describe in detail different optimized aggregation protocols of CsgA fibrillation depending on the final application of the fibrils. By implementing these protocols, the aggregation of CsgA is easily analyzed and mature fibrils obtained in a quantitative fashion, leaving insignificant amounts of monomer in solution.

5.2.1 Aggregation protocol for kinetic analysis

1. Prepare 100 mL 50 mM Tris/HCl pH 7.4 and filter through a 0.22 μm filter.
2. Use a PD-10 desalting column to reduce the amount of GdmCl and Imidazole as follows:
   - Elute the buffer.
   - Fill the column with MQ water and elute (2–3 times)
   - Equilibrate the column with 50 mM Tris/HCl pH 7.4; fill it and elute (2–3 times)
   - Add 1 mL of your protein solution.
   - Elute and collect flowthrough.
   - Add 1.5 mL 50 mM Tris/HCl pH 7.4.
   - Collect the protein in the next 1.5 mL 50 mM Tris/HCl pH 7.4 (aliquots of 250-500 μL)
   - Wash the column and store at 4 °C in 20% EtOH.
3. Measure protein concentration in the Nanodrop using ε_{280} = 11,460 $M^{-1}cm^{-1}$ and a protein molecular weight 15,049.15 Da.
4. Prepare the 96-well half area plate as follows to a final volume of 75 μL/well:
   - Add 6 μL of Th-T 500 μM to a final concentration of 40 μM.
   - Add 69 μL of 50 mM Tris/HCl pH 7.4 and CsgA to obtain a final concentration of 15 μM CsgA in 75 μL.
5. Set the FLUOstar to 37 °C.
6. Set up the aggregation protocol as follows:
   - Multichromatic: 1
   - Exc. Filter: 448–10
   - Em. Filter: 482–10
   - Gain: 900
   - Well scan: spiral avg.; ø 5 mm
   - Bottom optic
   - Settle time: 0 s
   - Kinetic window: 1
   - Number of cycles: 1000
   - Measurement start time: 0

- Number of flashes: 35
- Cycle time: 600 s
- Shake between readings: orbital; 0 rom; 0 s ON/OFF time
- Layout: index start value 1; increase; 1 replicate; horizontal

Note 1: If you are performing a seeded polymerization, recover freshly produced fibrils in an Eppendorf tube to obtain a minimum of 100 μL. Sonicate three times or 30 s (10 s ON, 10 s OFF) at 20% amplitude and incubate on ice for 1 min to reduce heating. Repeat it twice to a total of 3 sonication cycles and keep it on ice. Seeds should be prepared right before being used.

Note 2: Seeds should be added to 5% (μM/μM) of sonicated and non-sonicated fibrils previously formed.

5.2.2 Aggregation protocol for catalytic assays

1. Repeat steps 1–3 from 5.2.1.
2. In a 1.5 mL Eppendorf prepare 750 μL of CsgA at 40 μM in 50 mM Tris/HCl pH 7.4.
3. Add parafilm to avoid contaminations.
4. Incubate for 72 h at 37 °C under quiescent conditions.

5.3 FapC aggregation

As commented before, the fibrillation in *Pseudomonas* is also a thoroughly controlled mechanism that requires the combination of multiple factors *in vivo*. Nonetheless, when performed *in vitro* the major fap fibrillar component, FapC, readily forms mature and homogenous fibrils in a broad range of buffers, pH values, and salt concentrations. Here, we describe simple but optimized aggregation protocols that ease the kinetic analysis and the obtention of relevant amount of fibrils for further assays. As with CsgA the high aggregation capacity of FapC leads to an almost complete incorporation of monomers into fibrils. The aggregation protocols for kinetic analysis and catalytic assays for FapC are identical to those for CsgA (Sections 5.2.1–5.2.2) except to use FapC's extinction coefficient at 280 nm of 10095 $M^{-1}cm^{-1}$ and a protein molecular weight 24,998 Da.

5.4 Tau2N4R aggregation

The aggregation of the different tau isoforms in neuronal cells is associated with numerous neurological diseases, including Alzheimer's. This process is particularly slow, might take years, and is associated with posttranslational modifications such as hyperphosphorylation. Moreover, the process is

extremely complex and involves the formation of multiple toxic species. Similarly, the *in vitro* fibrillation of tau is performed by mimicking the hyperphosphorylated state upon the addition of anionic cofactors as heparin or anionic polysaccharides; to note few tau aggregation protocols in absence of cofactors have been described so far. Interestingly, structural analysis has demonstrated that tau protein might lead to the formation of multiple conformation both *in vitro* and *in vivo*, regardless of the spliced variant. Here, we detail a heparin-dependent aggregation protocol for Tau2N4R that has permitted low-resolution characterization of the fibril and aggregation process in periods shorter than 48 h. Moreover, the protocol is easily adapted to employ alternative cofactors and obtain a more homogenous conformation through the addition of seeds. Nevertheless, the yield of fibril formation is significantly lower than the one in functional amyloids and further isolation steps are required.

5.4.1 Standard aggregation protocol

1. Prepare 100 mL Tris/HCl 20 mM, TCEP 1 mM pH 7.4 and filter through a 0.22 μm filter.
2. Use a PD-10 desalting column to reduce the amount of EDTA and NaCl; follow the steps below:
   - Elute the buffer.
   - Fill the column with MQ water and elute (2-3 times).
   - Equilibrate the column with Tris/HCl 20 mM, TCEP 1 mM pH 7.4; fill it and elute (2-3 times).
   - Add 1 mL of your protein.
   - Elute and recover flowthrough.
   - Add 1.5 mL Tris/HCl 20 mM, TCEP 1 mM pH 7.4 to elute your protein.
   - Recover the protein in 1.5 mL Tris/HCl 20 mM, TCEP 1 mM pH 7.4.
   - Wash the column and keep it at 4 °C in EtOH 20%
3. To determine protein concentration, use a BCA (bicinchoninic acid) kit for reducing conditions and follow the instructions. Briefly:
   - Prepare BCA standards.
   - Prepare 200 μL of 20 mM Tris/HCl pH 7.4 in presence and absence of 1 mM TCEP (blanks).
   - Dilute competent reagent in a 1:1 dilution of MQ water:reagent buffer.
   - Prepare working solution 50:1 buffer A and buffer B.
   - Add 25 μL of blanks, standards and samples to independent Eppendorfs.
   - Add 25 μL of competent reagent, mix carefully and incubate at 37 °C for 15 min.

- Add 1 mL of working solution to each sample and incubate at 37 °C for 30 min.
- Leave 5-10 min at R.T.
- Add 200 μL of the samples to each well (in duplicate).
- Measure ABS at 562 nm.

4. Prepare the 96-wells half area plate as follows to a final volume of 75 μL/well:
   - Add 6 μL of 500 μM Th-T (final concentration 40 μM).
   - Add Tris/HCl 20 mM, TCEP 1 mM pH 7.4
   - Add Tau 2N4R to a final concentration of ≥ 10 μM.
   - Add 1.64 μL of Heparin at 1 mg/mL to a final ratio of 1:4 heparin:Tau (final concentration of 1.25 μM)
5. Set the FLUOstar to 37 °C.
6. Set up the aggregation protocol as follows:
   - Multichromatic: 1
   - Exc. Filter: 448–10
   - Em. Filter: 482–10
   - Gain: 900
   - Well scan: spiral avg.; ø 5 mm
   - Bottom optic
   - Settle time: 0 s
   - Kinetic window: 1
   - Number of cycles: 1000
   - Measurement start time: 0
   - Number of flashes: 35
   - Cycle time: 600 s
   - Shake between readings: orbital; 300 rpm; 0 s ON/OFF time
7. Introduce the plate and start the protocol.

   Note 1: Adjust the pH after TCEP addition.

   Note 2: Depending on the purification yield; once the protein is eluted, use an Amicon 10 kDa to increase Tau concentration. Add 500 μL of desalted Tau in each Amicon 10 kDa and centrifuge for 1 min at 12,600 g. If necessary, repeat the process. To recover your sample, turn the Amicon 10 kDa in a new tube and spin for 30 s

   Note 3: To increase the lifespan of a PD-10 desalting column save it at 4 °C in EtOH 20% in horizontal position to avoid resin compaction.

   Note 4: When employed for catalysis do not add Th-T; some wells should contain Th-T to validate the aggregation process.

   Note 5: If you are performing a seeded polymerization (which accelerates the process and homogenize the fibril sample), recover freshly produced

fibrils in an Eppendorf tube to obtain a minimum of 100 μL. Sonicate three times or 30 s (10 s ON, 10 s OFF) at 20% amplitude and incubate on ice for 1 min to reduce heating. Repeat it twice for a total of 3 sonication cycles and keep it on ice. Seeds should be prepared right before being used.

Note 6: Seeds should be added at a final 1% μM/μM of sonicated fibrils previously formed.

Note 7: The addition of seeds tends to homogenize the fibrillar content, leading to a major or dominant conformation.

5.5 α-Syn aggregation

As Tau2N4R, α-Syn aggregation is associated with several neurodegenerative diseases such as PD. Also, the fibrillation process is significantly slow and comprises multiple and alternative structures but, contrary to tau, it does not require posttranslational modifications. Over the years, several *in vitro* aggregation protocols have been described and most of them converge into the addition of beads and shaking conditions. The presence of beads and the shaking shorten the aggregation, resulting in mature fibrils in less than 48 h. Nevertheless, the process is highly influenced by environmental factors, such as pH and salt content, and different architectures could be generated. In the following sections we discuss standard aggregation protocols for the generation of multiple conformations, which permit structure-dependent characterization. As described above, the fibril content may be homogenized upon the addition of seeds, but further steps would be needed to isolate mature fibrils from the remaining soluble structures.

5.5.1 Standard aggregation protocol

1. Prepare PBS 1X and filter through a 0.22 μm filter.
2. Resuspend the lyophilized protein by adding 2.75 mL of PBS 1X
3. Let the protein re-hydrate for at least 5 min.
4. Gently resuspend by pipetting.
5. Filter the solubilized sample using a 0.22 μm filter.
6. Measure protein concentration in the Nanodrop using α-Syn's extinction coefficient at 280 nm of 5960 $M^{-1}cm^{-1}$ and a protein molecular weight 14460 Da.
7. Prepare the 96-well plate as follows to a final volume of 150 μL/well:
   - Add a sterile crystal bead of 1–2 mm diameter.
   - Add 12 μL of Th-T 500 μM to a final concentration of 40 μM.
   - Add PBS 1X
   - Add α-Syn to a final concentration of 70 μM.

8. Set the FLUOstar to 37 °C.

9. Set up the aggregation protocol as follows:

- Multichromatic: 1
- Exc. Filter: 448–10
- Em. Filter: 482–10
- Gain: 800
- Well scan: spiral avg.; ø 4 mm
- Bottom optic
- Settle time: 0 s
- Kinetic window: 1
- Number of cycles: 1000
- Measurement start time: 0
- Number of flashes: 30
- Cycle time: 300 s
- Shake between readings: double orbital; 300 rpm; 0 s ON/OFF time

  Note 1: Protein resuspension should be performed with extreme care to avoid bubbles and induce the formation of aggregates.

  Note 2: During the lyophilization and resuspension processes, some aggregates or structures could be induced making filtering process crucial.

  Note 3: Keep the protein on ice while unused to avoid aggregates formation.

  Note 4: The addition of the bead is crucial as it reduces the aggregation time by enhancing the mixing and acting as a seeding surface; it could be substituted by a 1/8″ diameter Teflon polyball (Polysciences Europe GmbH).

  Note 5: If you are performing a seeded polymerization (which accelerates the process and homogenize the fibril sample), recover freshly produced fibrils in an Eppendorf tube to obtain a minimum of 100 μL. Sonicate three times or 30 s (10 s ON, 10 s OFF) at 20% amplitude and incubate on ice for 1 min to reduce heating. Repeat it twice to a total of 3 sonication cycles and keep it on ice. Seeds should be prepared right before being used.

  Note 6: Seeds should be added at a final 1% μM/μM of sonicated fibrils previously formed.

  Note 7: The addition of seeds could be used to homogenize the fibrillar content leading to a major or predominant conformation.

  Note 8: When employed for catalysis do not add Th-T; some wells should contain Th-T to validate the aggregation process.

5.5.2 Aggregation protocol for generating alternative α-Syn conformations

1. Prepare PBS 1X and filter through a 0.22 μm filter.
2. Resuspend the lyophilized protein to a final concentration of 210 μM by adding PBS 1X
3. Let the protein re-hydrate for at least 5 min.
4. Gently resuspend by pipetting.
5. Use MQ water to re-hydrate a dialysis membrane of 3.6 kDa pores.
6. Add 2.75 mL of PBS resuspended α-Syn.
7. Dialyze in a 1:1000 (v/v) ratio in the presence of 50 mM Tris-HCl pH 7.5, or 50 mM Tris-HCl pH 7.5 supplemented with 150 mM KCl for 24 h.
8. Filter the protein through a 22-μm membrane to eliminate small aggregates of α-Syn.
9. Measure protein concentration in the Nanodrop using the extinction coefficient 5960 $M^{-1}cm^{-1}$ and protein molecular weight 14,460 Da.
10. Prepare the 96-well plate as follows to a final volume of 150 μL/well:
    - Add a sterile crystal bead of 1–2 mm diameter.
    - Add 12 μL of Th-T 500 μM to a final concentration of 40 μM.
    - Add the corresponding buffer (50 mM Tris-HCl pH 7.5 alone or supplemented with 150 mM KCl).
    - Add α-Syn to a final concentration of 70 μM.
11. Set the FLUOstar to 37 °C.
12. Set up the aggregation protocol as follows:
    - Multichromatic: 1
    - Exc. Filter: 448–10
    - Em. Filter: 482–10
    - Gain: 800
    - Well scan: spiral avg.; ø 4 mm
    - Bottom optic
    - Settle time: 0 s
    - Kinetic window: 1
    - Number of cycles: 1000
    - Measurement start time: 0
    - Number of flashes: 30
    - Cycle time: 300 s
    - Shake between readings: double orbital; 300 rpm; 0 s ON/OFF time

    Notes 1–6 and 8: See notes to 5.5.1.

    Note 9: As the resuspension process of a lyophilized sample might lead to the formation of aggregated species, it is crucial to do it with

the same buffer (PBS 1X) for all the samples, so the starting points are equivalent. The resuspension with the different buffers might induce alternative species that could impact the aggregation process; moreover, PBS 1X ensure a easy and efficient resuspension while Tris-based and other types of buffer might result in a loss of protein.

6. Aggregate validation and isolation protocols

To evaluate the catalytic capacity of amyloid fibril, it is essential to produce them in the absence of dyes that could remain bound and thus interfere with the interaction to the substrate. However, the formation of the fibrils must be previously validated. In the upcoming sections multiple characterization methods are described. Among the large amounts of potential methods, we focus on those easiest to implement, such as the use of an amyloid-specific dye. Nevertheless, as some fibrils might exhibit a poor affinity for these dyes, we also characterize orthogonal methods to ensure the formation of aggregates (light-scattering), to perform structural comparison and to separate fibrils from monomeric, oligomeric and/or protofibrillar species.

6.1 Materials, media, and buffer preparations

6.1.1 Reporters' stocks

1. Thioflavin-T (Th-T) 500 μM:
   - Add 3.19 mg Th-T
   - Add 12 mL MQ water.
   - Mix using a vortexer.
   - Add MQ water to make 20 mL
   - Filter through a 0.22 μm membrane
   - Make aliquots and keep at −20 °C until use.

6.2 Thioflavin-T measurements

Th-T has become the gold standard reporter on the amyloid field as it is considered an amyloid-specific dye (Malmos et al., 2017). When bound to the fibrils, the compound undergoes a conformational rearrangement that shifts its fluorescent excitation and emission maxima, leading to a significant increase in the fluorescence signal. The protocol described below enables a relatively sensitive validation of the presence of amyloid structures with low sample requirements.

1. Turn on the Cary Eclipse fluorimeter for at least 15 min to warm up the lamp.
2. Use the multicell holder and measure each well of the plate individually (if possible).
3. In an Eppendorf, make a 1:2 dilution of your fibril sample with 40 μM of Th-T.
4. Place 70 μL of the sample with Th-T into a quartz cuvette.
5. Set the following parameters with the multicell holder to measure Th-T fluorescence:
   - Data mode: Fluorescence
   - Scan mode: Emission
   - X Mode: Wavelength (nm)
   - Start (nm): 445
   - Stop (nm): 460
   - Ex, Wavelength (nm): 600
   - Ex, Slit (nm): 5
   - Em, Slit (nm): 5
   - Scan rate (nm/min): 600
   - Data interval (nm): 1
   - Averaging Time (s): 0.1000
   - Excitation filter: Auto
   - Emission filter: Open
   - PMT voltage (V): Medium
   - Corrected spectra: OFF
   - Multicell holder: Multicell
   - Multi zero: OFF
   - Method Name: Default
6. Transfer the sample to an Eppendorf and clean the cuvette using MQ water (add water 2-3 times).
7. When finished, clean the cuvette thoroughly and dry it.
   Note 1: The excitation and emission slit should be adjusted according to sample fluorescence.
   Note 2: Triplicates for each excitation wavelength must be performed to rule out precipitation during measurement.

6.3 Light-scattering analysis

The presence of aggregates, either amorphous or amyloids, may be also confirmed by non-specific techniques. Light-scattering constitutes a relevant and widely employed method to validate aggregate formation and perform

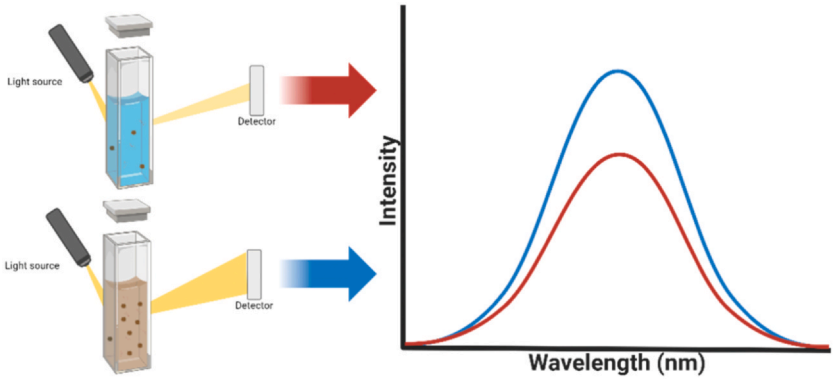

Fig. 3 Light-scattering overview. Schematic representation of light-scattering mechanisms and the potential results. As the number and size of particles (amyloids) increases (brown cuvette), the dispersion of the light becomes higher (blue). In contrast, the presence of small or scarce aggregates (blue cuvette) results in low or non-detectable signals (red).

comparative analysis. This technique analyzes the impact of particles in light dispersion and thus provides information on the number and size of the particles. Accordingly, an increased signal may be expected in the presence of fibrils (Fig. 3). Here, we describe an easy-to-implement light-scattering protocol that may be combined with the previous Th-T validation assay.

1. Turn on the Cary Eclipse for at least 15 min to heat the lamp.
2. Use the multicell holder and measure each well of the plate individually (if possible).
3. Transfer at least 60–70 μL of each sample to a quartz cuvette.
4. Set the following parameters with the multicell holder to measure light-scattering at 300 nm:
   - Data mode: Fluorescence
   - Scan mode: Emission
   - X Mode: Wavelength (nm)
   - Start (nm): 280
   - Stop (nm): 320
   - Ex, Wavelength (nm): 300
   - Ex, Slit (nm): 2.5
   - Em, Slit (nm): 5
   - Scan rate (nm/min): 600
   - Data interval (nm): 1
   - Averaging Time (s): 0.1000

- Excitation filter: Auto
- Emission filter: Open
- PMT voltage (V): Medium
- Corrected spectra: OFF
- Multicell holder: Multicell
- Multi zero: OFF
- Method Name: Default

5. Set the following parameters with the multicell holder to measure light-scattering at 340 nm:
   - Data mode: Fluorescence
   - Scan mode: Emission
   - X Mode: Wavelength (nm)
   - Start (nm): 320
   - Stop (nm): 360
   - Ex, Wavelength (nm): 340
   - Ex, Slit (nm): 2.5
   - Em, Slit (nm): 5
   - Scan rate (nm/min): 600
   - Data interval (nm): 1
   - Averaging Time (s): 0.1000
   - Excitation filter: Auto
   - Emission filter: Open
   - PMT voltage (V): Medium
   - Corrected spectra: OFF
   - Multicell holder: Multicell
   - Multi zero: OFF
   - Method Name: Default
6. Recover the sample to an Eppendorf and clean the cuvette using MQ water (add water 2-3 times).
7. When finished, clean the cuvette thoroughly and dry it.
   Note 1: The excitation and emission slit should be adjusted according to sample dispersion.
   Note 2: Triplicates for each excitation wavelength must be performed to discard precipitation during the process.

6.4 FTIR validation

Secondary structure is also a great indicator of amyloid fibrils, which in Fourier Transform Infared (FTIR) spectra give rise to a prominent peak corresponding to intermolecular β-sheets at 1624 cm^{-1} (Fig. 4).

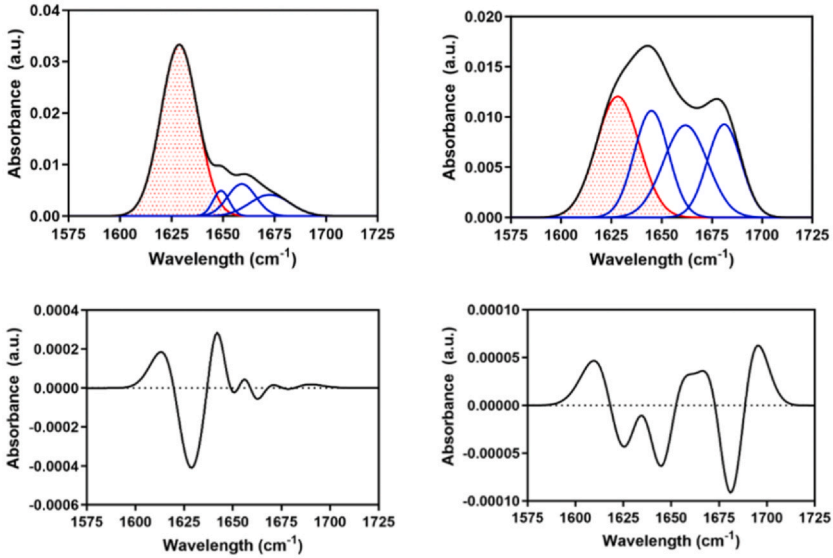

Fig. 4 Fourier-Transform InfraRed spectroscopy. Comparative analysis of the FTIR spectra (top) and second derivative (bottom) of different amyloids. In red the peak at 1624 cm^{-1}, indicative of intermolecular β-sheet.

1. Transfer 2 μL of the fibril suspension onto the measuring crystal on a Bruker Tensor 27 FTIR Spectrometer.
2. Dry under a gentle flow of N_2.
3. Repeat the process until 6 μL has been deposited.
4. Carefully close the Golden Gate MKII ATR accessory.
5. Measure at a spectral resolution of 4 cm^{-1}, within the 1800–1500 cm^{-1} range, performing 32 scans (Fig. 4).
6. Use the second derivative to assign the frequencies where the different components are located (Fig. 4).

   Note 1: It is essential that the drops remain in the lector when added and dried.

   Note 2: Do not completely close the Golden Gate MKII ATR accessory.

   Note 3: It is important to perform first the background before adding each sample.

   Note 4: Measuring while the sample is being added facilitates the control of the drying process and signal intensity.

6.5 Fibril centrifugation and monomer quantification

While functional amyloid such as CsgA and FapC exhibit almost a complete incorporation of the monomeric fraction intro the fibrillar structure, other amyloids are much less efficient. This is the case of several pathological amyloids as α-Syn or tau, whose aggregation leads to a low yield of monomer incorporation into the fibrils. Therefore, it is essential to isolate the fibrils and thus avoid the effect of other structures or the remaining monomers. Here we provide a simple protocol to exclusively precipitate amyloid fibrils.

1. Recover the fibrils into a 15 mL falcon tube.
2. Make aliquots in Eppendorf of equal volumes (*i.e.* 750 μL).
3. Centrifuge at 12,000 g for 30 min
4. Carefully recover the supernatant
5. Measure protein concentration on the recovered supernatant to calculate the remaining soluble (potentially monomeric) sample.
6. Calculate, in monomer equivalent, the theoretical concentration of amyloid fibrils.
7. Resuspend the fibrils into the desired buffer.

   Note 1: It is important to have the same volume, so every aliquot contains the same amount of fibrils after centrifugation.

   Note 2: Supernatant recovery should be performed with extreme care to avoid losing fibrils.

   Note 3: Fibril concentration could be also measured directly, as for the monomeric sample, but bear in mind that the absorption properties for different aggregated species might vary. This could be performed as an internal control, but not for comparative studies since different proteins or fibrillar conformation might lead to a different impact of the absorption properties.

   Note 4: This centrifugation step is crucial for pathological amyloids that present a low yield (≤ 50%) of monomer incorporation into the fibrils, but not for functional amyloids like CsgA or FapC, which exhibit barely complete incorporation.

   Note 5: The centrifugation of CsgA and FapC fibrils, unlike their pathological counterpart, leads to the formation of a labile, non-defined hydrogel. This might complicate its isolation as might retain the scarce remaining monomers. To solve this, CsgA and FapC fibrils should be frozen at −20 °C and then thawed, thus breaking the hydrogel architecture and releasing the fibrils.

7. Enzymatic screening protocols

The number of enzymatic activities that could be performed by an amyloid structure is overwhelming, and their characterization significantly time-consuming. Therefore, preliminary screening of large variety of catalytic activities of these fibrils is an essential first step to rule out unnecessary reactions. In this context, a precise selection of the reference substrate (Fig. 5) could facilitate the process and allow to screen multiple activities or fibrils in a single assay (Fig. 6). In the present section we provide a set of tools to easily set up screening protocols for catalytic amyloids. A list of reagents to use for these assays is provided in Table 3.

7.1 Materials, media, and buffers preparation

7.1.1 Buffer stocks for catalytic reactions

1. 1 M Tris/HCl pH 7.4
   - Add 121.14 g Tris BASE
   - Add MQ water to make 750 mL
   - Mix using a magnetic stirring.
   - Adjust pH using HCl to pH 7.4
   - MQ water to make 1000 mL
   - Filter through a 0.22 μm membrane

7.1.2 Chromogenic and fluorogenic substrate stocks

1. 4-Nitrophenyl acetate (pNPA) 40 mM
   - Add 7.43 mg of 4-Nitrophenyl acetate.
   - Add 1 mL of acetonitrile 100%
   - Mix gently by pipetting.
2. 4-Nitrophenyl phosphate ($pNPPO_4$) 40 mM
   - Add 8.76 mg of 4-Nitrophenyl phosphate.
   - Add 1 mL of 50 mM Tris/HCl pH 7.4
   - Mix gently by pipetting.
3. 4-Nitrophenyl butyrate (pNPB) 40 mM
   - Add 8.37 mg of 4-Nitrophenyl butyrate.
   - Add 1 mL of acetonitrile 100%
   - Mix gently by pipetting.
4. 4-Nitrophenyl palmitate (pNPP) 40 mM
   - Add 15.1 mg of 4-Nitrophenyl palmitate.
   - Add 1 mL of acetonitrile 100%

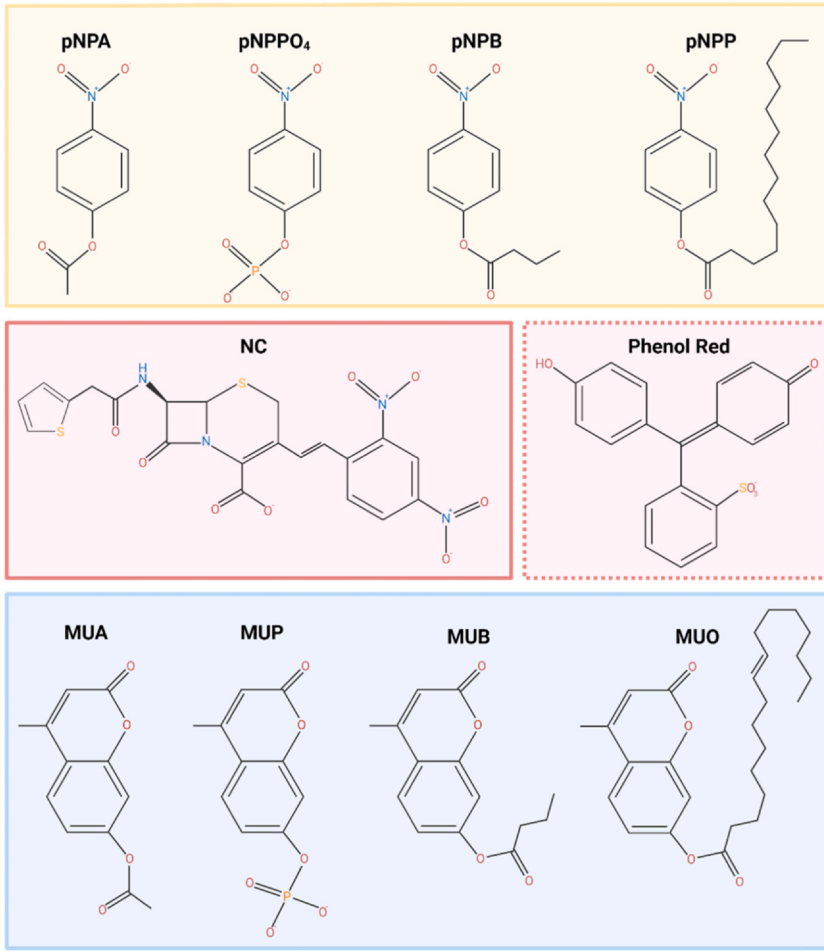

Fig. 5 Chemical structure of the catalytic substrates. Molecular representation of some of the most common substrates employed for catalytic assays. The yellow box indicates pNP derivates, red boxes correspond to substrates based on the absorbance and blue box indicates 4-MU derivates. Straight lines represent substrates that are directly targeted by the enzyme while dotted lines refer to indirect reporters of the catalytic activity.

- Mix gently by pipetting.
- Warm the sample at 37 °C until use.

5. Nitrocefin (NC) 40 mM
- Add 20.66 mg of Nitrocefin.
- Add 1 mL of acetonitrile 100%
- Mix gently by pipetting.

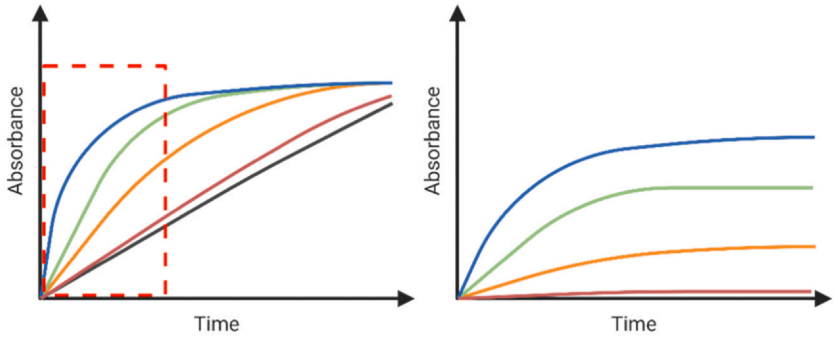

Fig. 6 Catalytic activity of different amyloid fibrils for absorbance-based substrates. Schematic representation of the catalytic capacity of different amyloids in a defined substrate. The left panel shows the raw data of the different fibrils (colors) and the substrate (black), which could undergo a complete degradation upon large periods of incubation. Right panel illustrates the substrate-normalized plot of the different fibrils in the first hours of the reaction.

6. 4-Methylumbelliferyl acetate (MUA) 40 mM
   - Add 8.73 mg of MUA.
   - Add 1 mL of acetonitrile 100%
   - Mix gently by pipetting.
7. 4-Methylumbelliferyl phosphate (MUP) 40 mM
   - Add 10.26 mg of MUP.
   - Add 1 mL of 50 mM Tris/HCl pH 7.4
   - Mix gently by pipetting.
8. 4-Methylumbelliferyl oleate (MUO) 40 mM
   - Add 17.62 mg of MUO.
   - Add 1 mL of DMSO 100%
   - Mix gently by pipetting.
9. 4-Methylumbelliferyl butyrate (MUB) 40 mM
   - Add 9.85 mg of MUB.
   - Add 1 mL of DMSO 100%
   - Mix gently by pipetting.
10. Phenol Red 2 mM
    - Add 35.44 mg of Phenol Red.
    - Add 30 mL of 50 mM Tris/HCl pH 7.4
    - Mix by vortexering.
    - Add 50 mM Tris/HCl pH 7.4 to make 50 mL

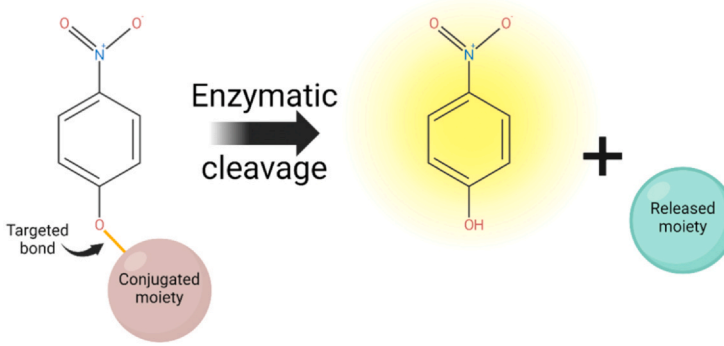

Fig. 7 Schematic representation of the spectrophotometric consequences of p-Nitrophenol based reactions.

11. Saturated CO_2
- Add 100 mL of MQ water in a bottle.
- Bubble CO_2 gas with a flow rate of 5 psi for 1 h under ice-cold conditions.

7.2 Screening with p-Nitrophenol-based probes

Some of the most used reporters for enzymatic activities are derivates of a phenolic compound called 4-Nitrophenol or p-Nitrophenol (pNP), which presents an intense yellow color at neutral and basic pH. pNP may be conjugated through the hydroxyl group with other functional groups such as acetate, phosphate groups or lipids. Depending on the group linked to pNP, different enzymatic activities such as esterase (acetate), phosphatase (phosphate group) or lipase (palmitate, butyrate, oleate) may be tested in parallel. In all cases, the catalytic activity releases the phenolic compound, resulting in a progressive increase in the absorption at 400–410 nm due to the increment of yellow coloring (Fig. 7). Here, we provide a step-by-step protocol that allows the screening of multiple enzymatic activities based on the use of this reporter.

1. Prepare 50 mM Tris/HCl pH 7.4

2. Set the CLARIOstar to 37 °C.

3. Set up the kinetic protocol as follows:
- Number of wavelengths: 1
- Wavelength (nm): 410
- Number of cycles: 61
- Cycle time: 180 s
- Shake 30–60 s before each cycle: double orbital; 100 rpm.

4. Prepare 10X aliquots of pNPA (esterase-like activity), $pNPPO_4$ (phosphatase-like activity) and pNPP or pNPB (lipase-like activity) diluted in 50 mM Tris/HCl pH 7.4.
5. Prepare the 96-well plate as follows:
   - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
   - Add fibrils (or buffer as reference) to a final concentration of 10 μM.
   - Add 15 μL of the substrate to a final 1X concentration.
6. Measure pNP generation at 410 nm for 3–5 h every 3 min.
7. Once finished, check the presence of yellow color in the samples to confirm the degradation of the tested substrate into pNP.
8. pNP-based substrates progressively self-degrade into pNP even in absence of fibrils, which means that in presence of amyloids higher levels or faster degradation needs to be detected to claim an enzymatic activity (Fig. 6).

   Note 1: Before starting a reaction, it is important to check pNP-based substrate solubility and stability at the diluted 10X concentration. Sometimes the change from acetonitrile to 50 mM Tris/HCl pH 7.4 leads to fast degradation of the substrate. Test the percentage of acetonitrile required to avoid degradation considering a final concentration in the plate not higher than 8%.

   Note 2: pNPA and pNPB exhibit reasonable solubility in 50 mM Tris/HCl pH 7.4 but still require small amounts of acetonitrile at concentrations higher than 10 mM.

   Note 3: Unlike other substrates, $pNPPO_4$ exhibits a high solubility and stability in 50 mM Tris/HCl pH 7.4 due to the existence of the polar phosphate group.

   Note 4: pNPP is extremely insoluble even in acetonitrile but heating the sample at 37 °C enhances its solubility. Alternatively, pNPP may be resuspended in an ethanol:chloroform solution (1:4) which is evaporated overnight; then, the sample is resuspended prior to the experiment in 50 mM HEPES buffer or 50 mM Tris/HCl containing 1% of Triton X-100 and finally dispersed upon sonication for 10 min.

   Note 5: A final concentration in the plate of 800 or 400 μM of the substrate should provide enough signal for detection. Higher concentrations may also be used.

   Note 6: Fibril theoretical concentration could also be modified either to a higher or lower extent. In any case, it is highly recommended to include wells containing the tested fibril concentration without the substrate to check if the absorbance increase is related to sample turbidity or incomplete aggregation.

   Note 7: Substrate references at the tested concentrations are essential since the substrate may self-degrade. Self-degradation should be used as a baseline to decipher fibril-mediated activity.

7.3 Other absorbance-based substrates, the case of nitrocefin and the lactamase-like activity

Unfortunately, some enzymatic activities cannot be screened or analyzed based on the above-described substrates. The lactamase reaction, namely the hydrolysis of the β-lactam ring found in many antibiotics, requires different substrates. A useful alternative is NC, a chromogenic cephalosporin substrate. The cleavage of the NC β-lactam ring induces a clear color shift from yellow to red that can be followed up by measuring the absorbance increase at 490 nm (Figs. 6 and Fig. 8). In the following section we describe a simplified method to detect lactamase-like activity in amyloid fibrils.

1. Prepare 50 mM Tris/HCl pH 7.4
2. Set the CLARIOstar to 37 °C.
3. Set up the kinetic protocol as follows:
   - Number of wavelengths: 1
   - Wavelength (nm): 490
   - Number of cycles: 61
   - Cycle time: 180 s
   - Shake 30–60 s before each cycle: double orbital; 100 rpm.
4. Prepare 10X aliquots of NC diluted in 50 mM Tris/HCl pH 7.4.
5. Prepare the 96-well plate as follows:
   - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
   - Add fibrils (or buffer as reference) to a final theoretical concentration of 10 μM.
   - Add 15 μL of the substrate to a final 1X concentration.
6. Measure NC degradation at 490 nm for 3 h every 3 min.
7. Once finished, check the presence of red color in the samples to confirm the degradation of the lactam ring of NC.
8. NC slowly autohydrolyses even in absence of fibrils, which means that in presence of amyloids higher levels or faster degradation needs to be detected to describe an lactamase-like activity.

   Note 1: Before starting a reaction, it is important to check NC solubility and stability at the diluted 10X concentration. Sometimes the change from acetonitrile to 50 mM Tris/HCl pH 7.4 leads to a fast degradation of NC. Test the percentage of acetonitrile required to avoid degradation, using a final concentration in the plate < 8%. Alternatively, NC may be resuspended in DMSO.

   Note 2: A final concentration in the plate of 400–800 μM of NC should provide enough signal for detection. Higher concentrations may also be used.

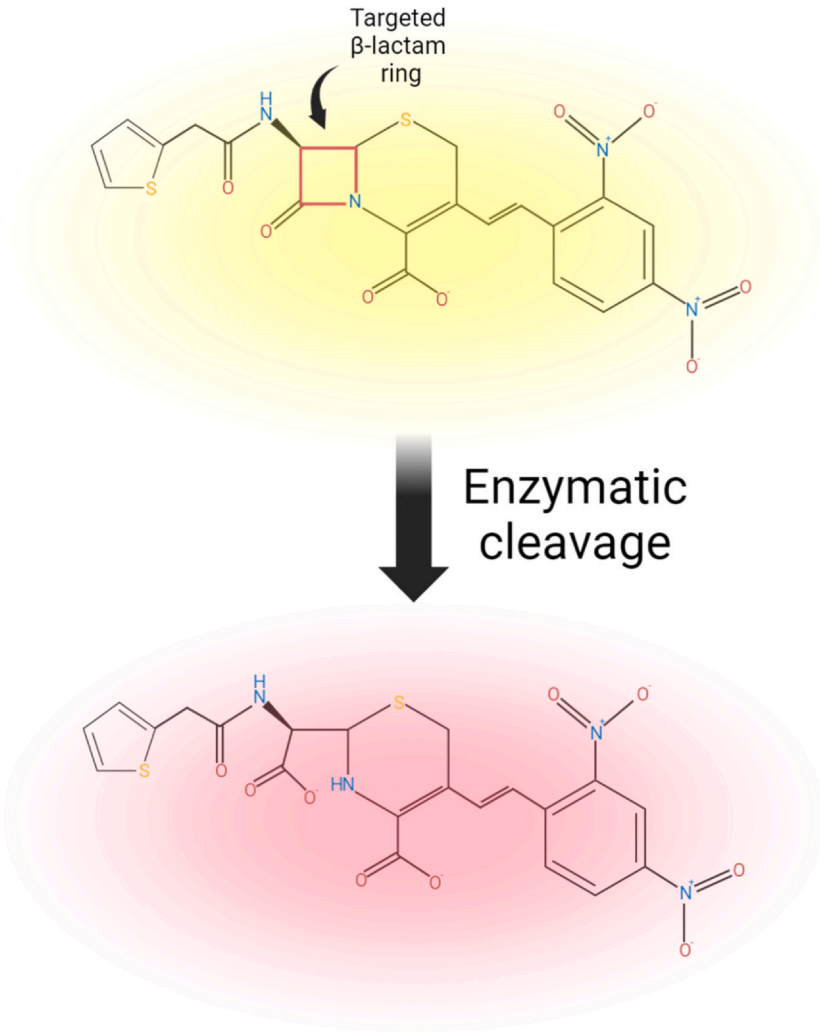

Fig. 8 Schematic representation of the Nitrocefin based reaction.

Note 3: Fibril theoretical concentration may also be modified either to a higher or lower extent. In any case, it is highly recommended to include wells containing the tested fibril concentration without the substrate to check if the absorbance increase is related to sample turbidity or incomplete aggregation.
Note 4: NC references at the tested concentrations are essential since the substrate could self-degrade. Thus, self-degradation should be used as a baseline to detect fibril-mediated activity.

7.4 Other absorbance-based substrates: phenol red and anhydrase-like activity

The aforementioned reporters, which are based on changes in the absorption, are also characterized by direct interaction within the potential catalyst. However, not all reactions are directly mimicked by a chromogenic substrate. Therefore, indirect reporters of catalytic activity based on environmental factors as pH should be also tested. The common pH indicator phenol red is a useful indirect reporter of anhydrase-like activity. Under basic conditions, phenol red presents a fuchsia coloration that progressively turns yellow in acidic conditions (Fig. 9). This facilitates the detection of carbonic anhydrase in amyloid fibrils, as it accelerates the conversion of CO_2 into carbonic acid in water and, therefore, promotes media acidification (Figs. 9 and Fig. 10). Some fibrils do not perform anhydrase-like activity while interacting with CO_2. This leads to a small pH decrease or to a slow recovery of the pH after CO_2 starts to evaporate (Fig. 10). Here, we describe an automatized method that overcomes the fast CO_2-mediated acidification at room temperature, providing robust kinetics.

1. Prepare 50 mM Tris/HCl pH 7.4 and 50 mM Tris/HCl pH 8.0
2. Set the kinetic protocol on the Varioscan plate reader as follows:
   - Group the replications on the Layout section.
   - Create multiple Area selections (depending on the number of samples) and select the wells to be measured.
   - Dispense protocol: inject 50 μL at a medium high speed.
   - Kinetic loop: 100 reads of 1 s
   - Absorbance wavelength (nm): 560
3. Prepare a stock of 100 nM of carbonic anhydrase in 50 mM Tris/HCl pH 7.4.
4. Prepare the 96-well plate as follows:
   - Add 42.5 μL of 50 mM Tris/HCl pH 8.0.
   - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
   - Add fibrils to a final theoretical concentration of 10 μM.
   - As a positive control, use 10 nM carbonic anhydrase.
   - Add 7.5 μL of 2 mM Phenol Red in 50 mM Tris/HCl pH 7.4
5. Using the injector, add 50 μL of CO_2-saturated MQ water.
6. Measure pH decrease by following phenol red color change at 560 nm for at least 100 s

   Note 1: MQ water saturation with CO_2 may be performed at room temperature, but the saturation yield is enhanced while on ice.

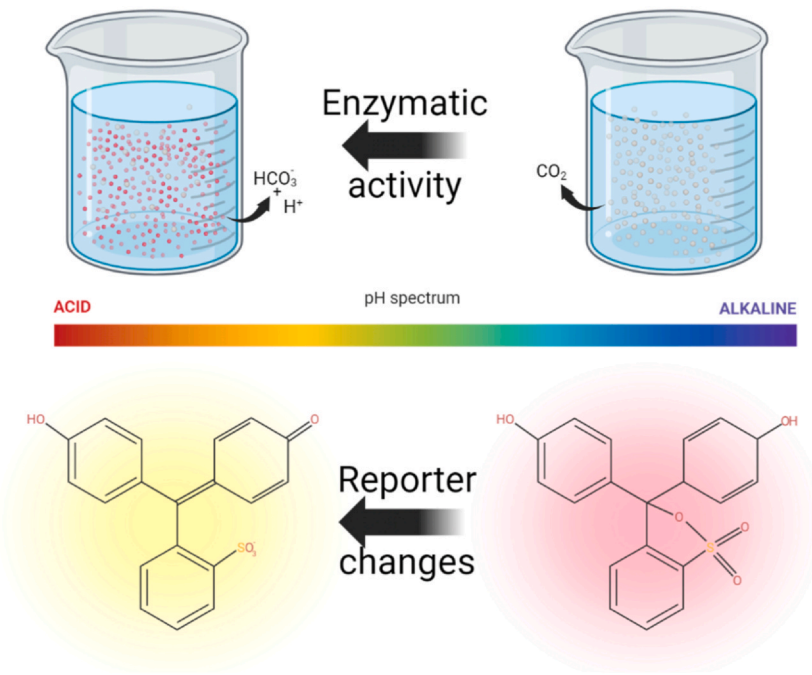

Fig. 9 Schematic representation of Phenol Red pH-dependent reaction.

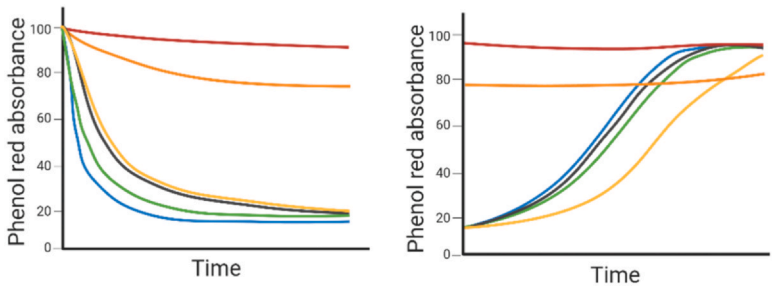

Fig. 10 Anhydrase-like activity of different amyloid fibrils. Phenol Red absorbance changes upon CO_2 addition in the presence of carbonic anhydrase (blue), different amyloid fibrils (green, yellow, and orange) and in their absence (black). Samples without CO_2 (red) are included as reference. Left panel: The addition of the carbonic anhydrase induces a fast equilibrium compared to the CO_2 control. Some fibrils (green) reproduce this activity, while others show no effect (yellow). Other fibrils interact with CO_2 but do not perform anhydrase activity, reducing pH variation (orange). Right panel: At longer periods of time, the evaporation of CO_2 induces a progressive recovery of pH. However, some fibrils induce a slower (yellow) or no (orange) recovery of the pH upon interaction with CO_2.

Note 2: A double control system based on the presence and absence of carbonic anhydrase must be applied to evaluate the existence of anhydrase-like activity (Fig. 10). The addition of CO_2 promotes a fast and natural pH acidification, which is enhanced in the presence of anhydrase-like enzymes.
Note 3: CO_2 may be manually added to the different wells, but it might lead to poorly defined kinetics due to the fast acidification process. In this case, it is highly recommended to perform sample analysis individually.
Note 4: Anhydrase-like activity is evident during the first 100–120 s; afterwards, there is a progressive evaporation of CO_2 which leads to a recovery of the pH and thus, to an increase in the absorption (Fig. 10). This could be also studied to analyze whether the fibrils are capable of retaining CO_2 by delaying the pH recovery (Fig. 10).
Note 5: Degassed MQ water may be used as a negative reference instead of CO_2-saturated water.
Note 6: By decreasing the temperature below 20 °C the reaction is significantly slowed down, facilitating comparison and analysis.

7.5 Fluorescent-based probes, 4-Methylumbelliferyl derivates

As alternative to the previously described probes based on absorbance variations, 4-Methylumbelliferyl (4-MU) fluorescent derivates should be considered. As with pNP based substrates, 4-MU derivates are conjugated through the hydroxyl group with other structures such as acetate, phosphate groups or lipids, thus reporting on different enzymatic activities. However, the cleavage of the bond releases the 4-MU subunit, increasing fluorescence signal at 490 nm, when exciting at 333 nm (Fig. 11). The following steps constitute a robust strategy that exploits 4-MU fluorescent properties at neutral pH to analyze the catalytic activity of amyloid fibrils.

1. Prepare 50 mM Tris/HCl pH 7.4
2. Set the CLARIOstar to 37 °C.
3. Set up the kinetic protocol as follows:
   - Multichromatic: 1
   - Exc.: 333–10
   - Em.: 490–10
   - Gain: 800
   - Well scan: spiral avg.; ø 4 mm
   - Bottom optic
   - Settle time: 0 s

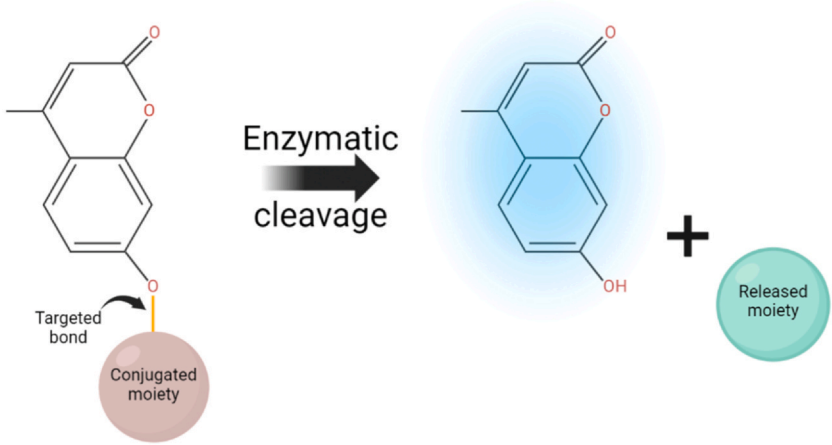

Fig. 11 Schematic representation of the 4-Methylumbelliferyl based reactions.

- Kinetic window: 1
- Number of cycles: 61
- Measurement start time: 0
- Number of flashes: 30
- Cycle time: 180 s
- Shake between readings: double orbital; 100 rpm; 30 s ON time

4. Prepare 10X aliquots of the 4-Methylumbelliferyl acetate, 4-Methylumbelliferyl phosphate, 4-Methylumbelliferyl oleate or 4-Methylumbelliferyl butyrate, diluted in 50 mM Tris/HCl pH 7.4.
5. Prepare the 96-well plate as follows:
   - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
   - Add fibrils (or buffer as reference) to a final theoretical concentration of 10 μM.
   - Add 15 μL of the substrate to a final 1X concentration.
6. Measure substrate degradation by exciting at 333 nm and measuring emission at 450 nm for 3 h every 3 min.
7. Substrates slowly self-degrade even in absence of fibrils, which means that in presence of amyloids higher levels or faster degradation needs to be detected to describe an enzymatic activity.
   Note 1: Before starting a reaction, it is important to check MU-based substrates' solubility and stability at the diluted 10X concentration. Sometimes the change from DMSO/acetonitrile to 50 mM Tris/HCl

pH 7.4 leads to a fast degradation of NC. Test the percentage of DMSO/acetonitrile required to avoid degradation considering a final concentration in the plate lower than 8%.

Note 2: Fibril theoretical concentration could also be modified either to a higher or lower extent. In any case, it is highly recommended to include wells containing the tested fibril concentration without the substrate to check if the absorbance increase is related to sample turbidity or incomplete aggregation.

Note 3: Substrates references at the tested concentrations are essential since the substrate may self-degrade. Thus, self-degradation should be used as a baseline to decipher fibril-mediated activity.

8. Characterizing the catalytic activity

Once the catalytic capacity of an amyloid fibril is established, a more detailed analysis is required. Such analysis might range from the standard Michaelis-Menten assay to more specific experiments including the effect of changes to reaction conditions or the surface/volume ratio among others. In the next sections, some protocols are provided to elucidate the complete catalytic potential of amyloid fibrils.

8.1 Materials, media, and buffers preparation

8.1.1 Buffer and substrate stocks for catalytic reactions

See Sections 7.1.1–7.1.2 (substrates 1–9).

8.2 Michaelis-menten analysis

The screening and discovery of catalytic potential within the amyloid scaffold open the door for quantitative characterization. One of the simplest analyses is based on Michaelis-Menten kinetics, which describes the rate of product formation as a function of the initial substrate concentration (Fig. 12). In the following lines we will describe step-by-step how to perform a Michaelis-Menten study on amyloid fibrils and hence characterize the affinity for the substrate, the catalytic constant and the specificity of the fibrils.

1. Prepare 50 mM Tris/HCl pH 7.4
2. Set the CLARIOstar to 37 °C.
3. Set up the kinetic protocol as indicated above for pNP-based, NC or 4-MU probes:

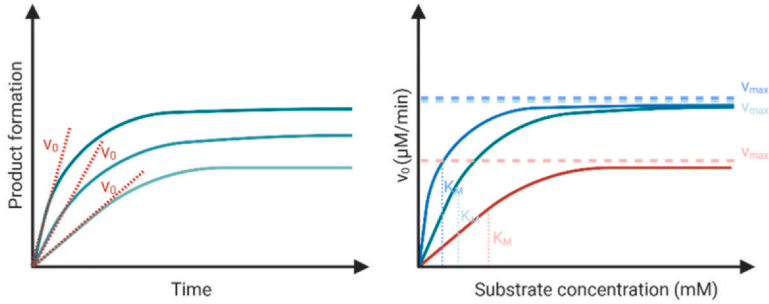

Fig. 12 Schematic for the collection of data for Michaelis-Menten characterization of catalytic amyloid. Left panel: Time profiles of the formation of product are collected at different time profiles (with autohydrolysis of substrate subtracted) and initial velocities determined. Right panel: Initial velocities are plotted *versus* substrate concentration to obtain K_M and v_{max}. Different curves represent different data from different fibrils.

4. Prepare 10X aliquots of the substrate, diluted in 50 mM Tris/HCl pH 7.4. Final concentration on the plate should range from 0.1 to 6 mM.
5. Prepare the 96-well plate as follows:
   - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
   - Add fibrils to a final theoretical concentration of 10 μM.
   - Add 15 μL of the substrate to a final 1X concentration.
6. Measure substrate degradation for 3 h every 3 min.
7. Substrates may slowly self-degrade even in absence of fibrils, which means that in the presence of amyloids, higher levels or faster degradation must be detected to describe an enzymatic activity.
8. Once the reaction is finished, calculate the concentration of product after subtracting its self-degradation.
9. Calculate the initial rates at different substrates concentrations during the first 30, 45 or 60 min either by simply considering the amount of product generated in the measured time or through the calculation of the slope when representing the amount of product at Y axis and the time at the X axis.
10. Plot the initial rates against their corresponding substrate concentration to obtain the K_M and the v_{max}.
11. Calculate the k_{cat} by using the obtained v_{max} and dividing it by the known fibril concentration.

12. Finally, calculate the catalytic efficiency as a relation between k_{cat} and K_M.
Note 1: Before starting a reaction, it is important to check substrates' solubility and stability at the diluted 10X concentration.
Note 2: Fibril theoretical concentration should be maintained constant and wells containing the tested fibril concentration without the substrate should be included to check if the absorbance increase is related to sample turbidity or incomplete aggregation.
Note 3: Substrate references at the tested concentrations are essential since the substrate could be self-degraded. Thus, self-degradation should be used as a baseline to detect fibril-mediated activity.
Note 4: Product concentration may be calculated from its molar extinction coefficient but would require previous calculations of the pathlength of the wells. It is simpler to establish a calibration curve with known concentrations.
Note 5: Due to the complex nature of the fibrils, the first 10 min of the reaction are often extremely noisy. Therefore, it is recommended to discard these first minutes from the constants analysis.
Note 6: To obtain a robust method to decipher the kinetic constants it is recommended to perform the analysis following the different methods described below (step 9).

8.3 Surface-dependent activity

Amyloid fibrils are morphologically characterized by a highly repetitive, long, and exposed surface. However, the exposed surface is reduced if the fibrils flocculate to form clumps. To address this, we use sonication to analyze how the variation of the ratio surface:volume impacts the catalytic activity by facilitating the accessibility of large substrate upon fibril fragmentation, or increasing the exposed surface through dismantling the fibrillar clusters (Fig. 13).

1. Prepare 50 mM Tris/HCl pH 7.4
2. Set the CLARIOstar to 37 °C.
3. Set up the kinetic protocol as indicated above for pNP-based, NC or 4-MU probes:
4. Prepare 10X aliquots of the substrate, diluted in 50 mM Tris/HCl pH 7.4.
5. Make aliquots of 200 μL of the fibril stock.
6. Sonicate the mature fibrils for 1 min (30 s ON, 30 s OFF) at 20% while on ice with a rod sonicator (Qsonica Sonicators).

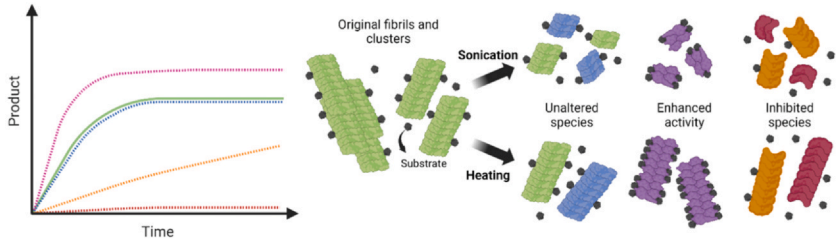

Fig. 13 Post-aggregational fibril modification. The catalytic activity of amyloid fibrils may be modified through sonication or heating of mature fibrils. As a result, fibrils or clusters may lead to free structures with similar activity (blue) or remain unaltered (green). The fibrils may also be modified to more active structures (violet) or completely (red) or partially (orange) lose their activity.

7. Prepare the 96-well plate as follows:
   - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
   - Add sonicated and non-sonicated fibrils to a final theoretical concentration of 10 μM.
   - Add 15 μL of the substrate to a final 1X concentration.
8. Measure substrate degradation for 3 h every 3 min.
9. Substrates may slowly self-degrade even in absence of fibrils, which means that in presence of amyloids higher levels or faster degradation needs to be detected to describe an enzymatic activity.

   Note 1: Before starting a reaction, it is important to check substrates solubility and stability at the diluted 10X concentration.

   Note 2: Fibril theoretical concentration should be maintained constant and wells containing the tested fibril concentration without the substrate should be included to check if the absorbance increase is related to sample turbidity or incomplete aggregation.

   Note 3: Substrates references at the tested concentrations are essential since the substrate could be self-degraded. Thus, self-degradation should be used as a baseline to decipher fibril-mediated activity.

   Note 4: Fibril sonication should be performed just before its addition to the plate to avoid the formation of clusters.

   Note 5: Sonicated fibrils should be kept on ice to avoid the formation of clusters that reduce the exposed areas.

   Note 6: Surface-depending impact could be further evaluated with different strains but also oligomeric and prefibrillar species.

8.4 Temperature impact

Amyloid fibrils generally resist extreme conditions, as for example, high temperatures on which globular protein could misfold. Despite this, the catalytic activity could be affected to different extents, such as a decrease upon fibril depolymerization or an increase by enhancing the accessibility to the catalytic site (Fig. 13). Here we explore the effect of high temperatures on the catalytic activity of the fibril.

1. Prepare 50 mM Tris/HCl pH 7.4
2. Set the CLARIOstar to 37 °C.
3. Set up the kinetic protocol as indicated above for pNP-based, NC or 4-MU probes:
4. Prepare 10X aliquots of the substrate, diluted in 50 mM Tris/HCl pH 7.4.
5. Make aliquots of 200 μL of the fibril stock.
6. Heat the mature fibrils at 95 °C for 10 min.
7. Let the sample cool down for no more than 5 min.
8. Prepare the 96-well plate as follows:
   - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
   - Add fibrils to a final theoretical concentration of 10 μM.
   - Add 15 μL of the substrate to a final 1X concentration.
9. Measure substrate degradation for 3 h every 3 min.
10. Substrates may slowly self-degrade even in absence of fibrils, which means that in presence of amyloids higher levels or faster degradation needs to be detected to describe an enzymatic activity.

    Note 1: Before starting a reaction, it is important to check substrates solubility and stability at the diluted 10X concentration.

    Note 2: Fibril theoretical concentration should be maintained constant and wells containing the tested fibril concentration without the substrate should be included to check if the absorbance increase is related to sample turbidity or incomplete aggregation.

    Note 3: Substrates references at the tested concentrations are essential since the substrate could be self-degraded. Thus, self-degradation should be used as a baseline to decipher fibril-mediated activity.

    Note 4: Sample heating and cool down should be performed just before its addition to the plate.

    Note 5: Letting the fibrillar sample cool down is essential as an increased temperature might affect the catalytic analysis.

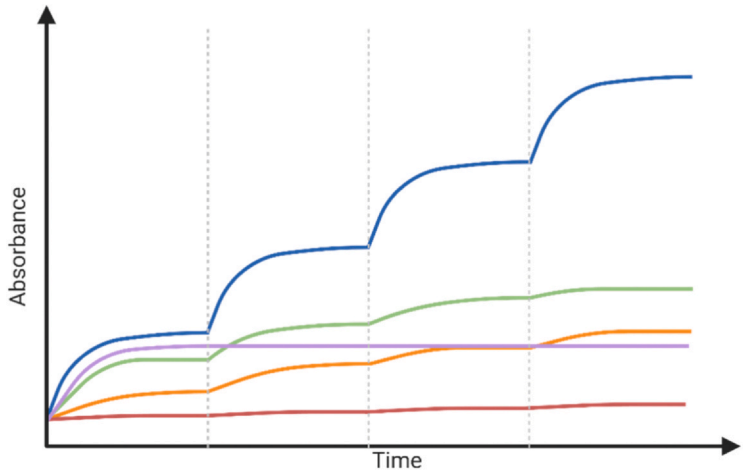

Fig. 14 Substrate-normalized fibril activity upon multiple additions of the substrate. The addition of more substrate at different time points (doted lines) facilitates the analysis of the generation of the catalytic site. As a result, some active fibrils show a constant regeneration of the catalytic site (blue and orange). However, some of the fibrils exhibit a complete lack of regeneration (purple) or a progressive loss of the activity (green) upon substrate addition. Non-active fibrils (red) would not display any activity rather than natural substrate degradation.

8.5 Recycling fibrils

The catalytic site of the enzymes might become compromised with time due to denaturation or irreversible binding of the substrate. The protocol described below addresses this concern by testing upon multiple additions of the substrate, whether the catalytic site is regenerated after binding to the substrate molecules and remains functional (Fig. 14).

1. Prepare 50 mM Tris/HCl pH 7.4
2. Set the CLARIOstar to 37 °C.
3. Set up the kinetic protocol as indicated above for pNP-based, NC or 4-MU probes:
4. Prepare 20X aliquots of the substrate, diluted in 50 mM Tris/HCl pH 7.4.
5. Prepare the 96-well plate as follows:
   - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
   - Add fibrils to a final theoretical concentration of 10 μM.
   - Add 7.5 μL of the 20X substrate to a final 1X concentration.

6. Measure substrate degradation for 5 h every 3 min.
7. Substrates may slowly self-degrade even in absence of fibrils, which means that in the presence of amyloids, higher levels or faster degradation needs to be detected to describe an enzymatic activity.
8. After 5 h, add another 7.5 μL of the 20X substrate to a final 1X concentration.
9. Measure substrate degradation for 5 h every 3 min.

   Note 1: Before starting a reaction, it is important to check substrates solubility and stability at the diluted 20X concentration.

   Note 2: Fibril theoretical concentration should be maintained constant and wells containing the tested fibril concentration without the substrate should be included to check if the absorbance increase is related to sample turbidity or incomplete aggregation.

   Note 3: Substrates references at the tested concentrations are essential since the substrate could be self-degraded. Thus, self-degradation should be used as a baseline to decipher fibril-mediated activity.

   Note 4: Substrate concentration should be previously defined by taking into consideration that the final concentration of the product will be double; therefore, the selected substrate concentration should not lead to the generation of sufficient product as to saturate the signal.

   Note 5: The addition of fresh substrate should lead to a secondary Michaelis-Menten profile if the catalytic residues of the fibrils remain accessible.

8.6 Measuring the activity of monomeric and prefibrillar species

Although the fibrils are previously isolated from the monomeric and prefibrillar fraction, it is important to analyze the catalytic potential of non-fibrillar species. The lack of activity in these non-fibrillar conformation would definitely demonstrate that the activity is associated to the fibril structure rather than to the protein sequence or alternative structures. Here we briefly describe an easy-to-implement validation process.

1. Prepare 50 mM Tris/HCl pH 7.4
2. Set the CLARIOstar to 37 °C.
3. Set up the kinetic protocol as indicated above for pNP-based, NC or 4-MU probes:
4. Prepare 10X aliquots of the substrate, diluted in 50 mM Tris/HCl pH 7.4.

5. Prepare monomeric protein as requested.
6. Prepare the 96-well plate as follows:
   - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
   - Add fibrils or monomers to a final theoretical concentration of 10 μM.
   - Add 15 μL of the substrate to a final 1X concentration.
7. Measure substrate degradation for 3 h every 3 min.
8. Substrates could slowly self-degrade even in absence of fibrils, which means that in the presence of amyloids higher levels or faster degradation needs to be detected to describe an enzymatic activity.
   Note 1: Before starting a reaction, it is important to check substrates solubility and stability at the diluted 10X concentration.
   Note 2: Fibril theoretical concentration should be maintained constant and wells containing the tested fibril concentration without the substrate should be included to check if the absorbance increase is related to sample turbidity or incomplete aggregation.
   Note 3: Substrates references at the tested concentrations are essential since the substrate could be self-degraded. Thus, self-degradation should be used as a baseline to decipher fibril-mediated activity.
   Note 4: The reaction conditions may induce the formation of amyloids during the process, which could mislead the interpretation of the results. The aggregation propensity should be taking into account.
   Note 5: Highly aggregation prone amyloids, as functional amyloids, might not remain monomeric under normal conditions for long periods of time. Therefore, they will be present in a protofibrillar or oligomeric conformation rather than a monomeric state.

8.7 Fibril adaptation

As commented before, the environmental conditions can significantly affect the architecture of the amyloid fibril. In this context, the exposure of the amyloid protein to a particular substrate can potentially lead to a fibrillar conformation with a higher affinity and, therefore, an enhanced catalytic capacity. Here we provide a simplified procedure that allows to analyze the impact on the catalytic potential of fibrillating in a substrate-rich environment.

1. Make amyloid fibrils as stated before (see Section 5), but in the presence or absence of the substrate that is going to be tested.
2. Prepare 50 mM Tris/HCl pH 7.4
3. Isolate upon centrifugation and wash the fibrils with 50 mM Tris/HCl pH 7.4 to discard the remaining substrate/product of the solution.

4. Validate amyloid formation (see Section 6).
5. Repeat the process 3 times.
6. Set the CLARIOstar to 37 °C.
7. Set up the kinetic protocol as indicated above for pNP-based, NC or 4-MU probes.
8. Prepare 10X aliquots of the substrate, diluted in 50 mM Tris/HCl pH 7.4.
9. Prepare monomeric protein as required.
10. Prepare the 96-well plate as follows:
    - Add 50 mM Tris/HCl pH 7.4 to a final volume of 150 μL.
    - Add fibrils to a final theoretical concentration of 10 μM.
    - Add 15 μL of the substrate to a final 1X concentration.
11. Measure substrate degradation for 3 h every 3 min.
12. Substrates may slowly self-degrade even in absence of fibrils, which means that in presence of amyloids higher levels or faster degradation needs to be detected to describe an enzymatic activity.

    Note 1: In addition to regularly formed fibrils, fibril should be also formed in absence of the substrate but in presence of the same buffer conditions to elucidate if the impact is due to the substrate or its buffer conditions.

    Note 2: During the aggregation process the substrate could have been transformed into the product.

    Note 3: It is essential to validate amyloid formation since the presence of substrate/product might impact the process.

    Note 4: Before starting a reaction, it is important to check substrates solubility and stability at the diluted 10X concentration.

    Note 5: Fibril theoretical concentration should be maintained constant and wells containing the tested fibril concentration without the substrate should be included to check if the absorbance increase is related to sample turbidity or incomplete aggregation.

    Note 6: Substrates references at the tested concentrations are essential since the substrate could be self-degraded. Thus, self-degradation should be used as a baseline to decipher fibril-mediated activity.

9. Bioinformatic analysis of the catalytic activity

Computational approaches allow us to pinpoint interactions between protein residues and a ligand, allowing a systematic understanding of

experimental observations and guiding subsequent tests. In the context of this work, this facilitates the strategic incorporation of an active site into a desired protein scaffold.

Computational grafting is commonly employed to search for a protein scaffold to harbor a functional motif, often a small peptide. This is particularly useful in immunology to transplant epitopes from one antigen to another protein. Our task however involves integrating an enzyme's active site into a specific scaffold, which is amyloid. This active site consists of multiple residues involved in substrate binding, stabilization of transition state and a typical Ser-His-Asp catalytic triad. Despite being distant in the enzyme's linear sequence, they converge in the three-dimensional space due to the enzyme's folding.

In this section we elaborate on our *in silico* approach which involves the construction of a reliable computational model; the analysis of the interactions between our model substrate and the native amyloid structure using molecular docking and MD simulations; strategic modification of the amyloid sequence to incorporate a mimic of an enzyme's active site and validation of the designed catalytic amyloid using high level Quantum mechanics/molecular mechanics (QM/MM) simulations.

9.1 Structure acquisition and homology modeling

The construction of a reliable computational model of the protein of interest is imperative for the success of any *in silico* approach. The search for a 3D structure should start on the Protein Data Bank (PDB) for a structure resolved with x-ray crystallography, NMR, or cryo-electron microscopy with good resolution. There are amyloid structures deposited on the PDB (Sleutel, Pradhan, Volkov, & Remaut, 2023; Wasmer, 2008; Bu, Dee, & Liu, 2024) but the number is scarce. Fortunately, computational algorithms to predict protein structures from the amino acid sequence are nowadays very reliable, some notable examples include SWISS MODEL (Waterhouse et al., 2018), RoseTTAFold (Baek et al., 2021) and AlphaFold2 (Jumper et al., 2021). The user should expect a reliable model from any of these algorithms, however, caution is advised for inherently disordered regions and domain-domain interfaces. The webserver AlphaFold2 has gained great popularity and can easily be run on any web browser on the google collaboration site ColabFold (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb) (Mirdita et al., 2022). The query sequence should be carefully curated by removing signal sequences that are not present in the mature structure and inherently disordered regions if they are known not to be involved. The construction of an oligomer is advised, otherwise important

intermolecular interaction sites may be missed. For this task, we recommend using a template that shares the same building blocks as the protein of interest, if available since computational algorithms tend to have difficulties to correctly model the interfaces. In the end, the user obtains the predicted structure in PDB format and a per-residue confidence score (pLDDT) between 0 and 100. A good model usually exhibits pLDDT scores above 80 for almost all residues.

9.2 Molecular dynamics simulations

Evaluating the stability and dynamics of the amyloid structure is essential, as is ensuring extensive coverage of the conformational space for downstream analyses. There is a panoply of software packages that make use of Newtonian equations of motion to perform Molecular Dynamics simulations in systems up to millions of atoms. AMBER (Case et al., 2023), GROMACS (Abraham et al., 2015) and NAMD (Phillips et al., 2020) are some of the most acclaimed ones and are freely available for academic research.

Here we describe the protocol we followed using GROMACS 2022, employing the AMBER ff19SB force field. This should work for most cases. Replicates are recommended to enhance the exploration of the conformational space.

9.2.1 Software requirements

GROMACS can be installed on Linux, Windows, and MAC OS operating systems. The current protocol was optimized on a Linux Mint distribution. The installation of ANTECHAMBER package is also required to follow this protocol to generate the topology file. We recommend the freely available Visual molecular dynamics (VMD) for visualizing the resulting trajectories (Humphrey, Dalke, & Schulten, 1996). The ParmEd package will be needed to convert the AMBER topology to GROMACS format (https://github.com/ParmEd/ParmEd).

9.2.2 Protocol

9.2.2.1 Protonation of the protein

This should be done based on pK_a estimation of titratable residues (Asp, Glu, Lys, His) using tools such as PROPKA or H++ and chemical intuition by visualizing the structure. For complex cases, other, more accurate and much more expensive, methods such as constant pH MD are available. For the task at hand, we chose PROPKA (Olsson, Søndergaard, Rostkowski, & Jensen, 2011; Søndergaard, Olsson, Rostkowski, & Jensen, 2011). This software can be installed locally but can also be easily run on webservers such as ProteinPrepare (Martínez-Rosell, Giorgino, & De Fabritiis, 2017).

The output is a list of estimated pK_a values for each titratable residue. If the estimations point to a protonation state different from the physiological ones, the user should visually inspect the result and change on the pdb file the residue names to match the desired protonation state on the force field used. Since we are using an amber force field the possible changes are ASP to ASH (neutral aspartate); GLU to GLH (neutral glutamate); HIS (neutral histidine) to either HID, HIE or HIP (delta protonated histidine, epsilon protonated histidine and positively charged histidine respectively) and LYS to LYN (neutral lysine).

9.2.2.2 Generation of the topology file

On this stage we will use LEaP, available on the ANTECHAMBER package.

This stage should start with the cleaning of the pdb file. Unnecessary information should be removed, leaving only the 3D coordinates of the protein; if the structure is a multimer, a line with a "TER" instruction should be added to tell LEaP it should not connect the two adjacent residues, and the names of the residues should match the desired protonation states as described above. We recommend the use of pdb4amber script to obtain a renumerated and ready to use pdb file. It also informs on the presence of structural cysteine sulfur bridges. The command is `pdb4amber -i original.pdb -o renumerated.pdb`.

Use the tleap input below to protonate the protein, generate the final topology and coordinates files with the ff19SB forcefield (Tian et al., 2020), neutralize and solvate the system with either Cl^- or Na^+ ions and a 15.0 Å water box using the OPC3 water model (Izadi and Onufriev, 2016). The input can be run using either tleap or xleap scripts with the following command: `tleap -f LeaP_input.in`.

```
source leaprc.protein.ff19SB
source leaprc.water.opc3
mol = loadpdb renumerated.pdb
savepdb mol Amyloid_dry.pdb
saveamberparm mol Amyloid_dry.prmtop Amyloid_dry.crd
solvateBox mol OPC3BOX 15.0
addions mol Na+ 0
addions mol Cl- 0
savepdb mol Amyloid_solvated.pdb
saveamberparm mol Amyloid_solvated.prmtop Amyloid_solvated.crd
quit
```

After running the LeaP you should get a `(no restraints)` message. Check for warnings on the output nonetheless. Then use ParmEd to convert the files from AMBER to GROMACS format, instructions can be found at https://github.com/ParmEd/ParmEd.

9.2.2.3 Energy minimization

During this step, we will remove eventual steric clashes that may be introduced during the modelling, protonation and solvation steps. Typically, a two-step minimization is followed in GROMACS, the first using the steepest descent algorithm and the second using the conjugate gradient algorithm. Typical inputs are below.

Min1.mdp

```
title       = Min1
integrator  = steep
emtol       = 1000.0
emstep      = 0.01
nsteps      = 3000
cutoff-scheme   = Verlet
ns_type         = grid
rlist           = 1.0
coulombtype     = PME
rcoulomb        = 1.0
rvdw            = 1.0
pbc             = xyz
```

Min2.mdp

```
title       = Min1
integrator  = cg
emtol       = 500.0
emstep      = 0.01
nsteps      = 5000
nstcgsteep = 1000
cutoff-scheme   = Verlet
ns_type         = grid
rlist           = 1.0
coulombtype     = PME
rcoulomb        = 1.0
rvdw            = 1.0
pbc             = xyz
```

.

These inputs can be run using the grompp module of GROMACS to generate a single binary input file, and the mdrun engine, using the following commands in sequence:

```
gmx grompp -f Min1.mdp -c Amyloid_solvated.gro -p Amyloid_solvated.top
-o Min1.tpr
gmx mdrun -v -deffnm Min1
gmx grompp -f Min2.mdp -c Min1.gro -p Amyloid_solvated.top -o Min2.tpr
gmx mdrun -v -deffnm Min2
```

9.2.2.4 System equilibration and production run

In this step we will heat and equilibrate the system at the desired temperature. The equilibration is done with restraints on the backbone atoms to avoid unreasonable motions. Then, a production run will be carried out with all atoms free to move. The use of replicates is advised to efficiently explore conformational space.

For this step, we will need to make use of the make_ndx and genrestr modules of GROMACS. The `gmx make_ndx -f Amyloid_solvated.gro` command will generate an index.ndx and the `echo "Backbone" | gmx genrestr -f Amyloid_solvated.gro -n index.ndx -o posres_BB.itp -fc 500 500 500` command will generate a posres_BB.itp file with instructions to apply a harmonical positional restraint of 500 kJ/mol/nm^2 on every backbone atom of the protein.

Open the Amyloid_solvated.top file. After all the information related to the protein, add the following lines:

```
#ifdef POSRES
#include "posres_BB.itp"
#endif
```

Then, an equilibration with the NVT ensemble will be run to bring the system up to the production temperature, followed by a second equilibration with the NPT ensemble to equilibrate the system at actual experimental conditions. Both are run with restraints on the backbone atoms to prevent unrealistical structural deformations. Typical inputs and run commands are below. If you are running on an HPC, refer to the cluster documentation to check the most efficient way to launch mdrun given the HPC architecture.

NVT_eq.mdp

```
title                    = 100ps NVT Heating MD
define                   = -DPOSRES
integrator               = md
nsteps                   = 50000
dt                       = 0.002
nstxout-compressed       = 500
nstvout                  = 500
nstenergy                = 500
nstlog                   = 500
continuation             = no
constraint_algorithm     = lincs
constraints              = h-bonds
lincs_iter               = 1
lincs_order              = 4
cutoff-scheme            = Verlet
ns_type                  = grid
nstlist                  = 10
vdwtype                  = cutoff
vdw-modifier             = Potential-shift-Verlet
rvdw-switch              = 0.9
rvdw                     = 1.0
DispCorr                 = EnerPres
coulombtype              = PME
rcoulomb                 = 1.0
pme_order                = 4
fourierspacing           = 0.125
tcoupl                   = V-rescale
tc-grps                  = non-Protein Protein
tau_t                    = 0.1        0.1
ref_t                    = 298.15     298.15
pcoupl                   = no
pbc                      = xyz
gen_vel                  = yes
gen_temp                 = 298.15
gen_seed                 = -1
annealing                = single single
annealing-npoints        = 2 2
annealing-time           = 0 100 0 100
annealing-temp           = 0 298.15 0 298.15
```

NPT_eq.mdp

```
title                    = 2ns NPT Equilibration
define                   = -DPOSRES
integrator               = md
nsteps                   = 1000000
dt                       = 0.002
nstxout-compressed       = 500
nstvout                  = 500
nstenergy                = 500
nstlog                   = 500
continuation             = yes
constraint_algorithm     = lincs
constraints              = h-bonds
lincs_iter               = 1
lincs_order              = 4
cutoff-scheme            = Verlet
ns_type                  = grid
nstlist                  = 10
rlist                    = 1
vdwtype                  = cutoff
vdw-modifier             = Potential-shift-Verlet
rvdw-switch              = 0.9
rvdw                     = 1.0
DispCorr                 = EnerPres
coulombtype              = PME
rcoulomb                 = 1.0
pme_order                = 4
fourierspacing           = 0.125
tcoupl                   = V-rescale
tc-grps                  = non-Protein Protein
tau_t                    = 0.1     0.1
ref_t                    = 298.15  298.15
pcoupl                   = Berendsen
pcoupltype               = isotropic
tau_p                    = 2.0
ref_p                    = 1.0
compressibility          = 4.5e-5
refcoord_scaling         = com
pbc                      = xyz
gen_vel                  = no
```

```
gmx grompp -f NVT_eq.mdp -c Min2.gro -r Min2.gro -p
Amyloid_solvated.top -n index.ndx -o NVT_eq.tpr
gmx mdrun -v -deffnm NVT_eq
gmx grompp -f NPT_eq.mdp -c NVT_eq.gro -r NVT_eq.gro -p
Amyloid_solvated.top -n index.ndx -o NPT_eq.tpr
gmx mdrun -v -deffnm NPT_eq
```

With an equilibrated system, we will conduct a production run without restraints. Below is an input example for a 200 ns simulation.

NPT_prod.mdp

```
title                    = 200ns NPT Production
integrator               = md
nsteps                   = 100000000
dt                       = 0.002
nstxout                  = 0
nstvout                  = 0
nstfout                  = 0
nstenergy                = 50000
nstlog                   = 50000
nstxout-compressed       = 50000
continuation             = yes
constraint_algorithm     = lincs
constraints              = h-bonds
lincs_iter               = 1
lincs_order              = 4
cutoff-scheme            = Verlet
ns_type                  = grid
nstlist                  = 10
rlist                    = 1.0
vdwtype                  = cutoff
vdw-modifier             = Potential-shift-Verlet
rvdw-switch              = 0.9
rvdw                     = 1.0
coulombtype              = PME
```

```
rcoulomb                = 1.0
pme_order               = 4
fourierspacing          = 0.125
tcoupl                  = V-rescale
tc-grps                 = non-Protein Protein
tau_t                   = 0.1     0.1
ref_t                   = 298.15  298.15
pcoupl                  = Parrinello-Rahman
pcoupltype              = isotropic
tau_p                   = 1.0
ref_p                   = 1.0
compressibility         = 4.5e-5
pbc                     = xyz
DispCorr                = EnerPres
gen_vel                 = no
```

```
gmx grompp -f NPT_prod.mdp -c NPT_eq.gro -p Amyloid_solvated.top -n
index.ndx -o NPT_prod.tpr
gmx mdrun -v -deffnm NPT_prod
```

9.2.2.5 Analysis of the simulation

After running the production, the temperature, pressure and energy of the system and the root mean square deviation (RMSd) of the backbone of the protein must be checked to ensure the system is equilibrated and no unrealistical movements have taken place.

First the periodicity of the system must be corrected. The following commands should work for most cases: `echo "4 0" |gmx trjconv -s NPT_prod.tpr -f NPT_prod.xtc -o NPT_prod_pbc.xtc -center -pbc mol -ur compact -n index.ndx`.

To calculate the RMSd of the backbone, use the following command: `echo "4 4" |gmx rms -s Amyloid_solvated.gro -f NPT_prod_pbc.xtc -o rmsd.xvg -tu ns`.

To obtain representative structures from the trajectories, the cluster module of GROMACS can be used. It is advised to consider only the frames on the equilibrated part of the simulation. The following example command can be used: `echo "4 1" |gmx cluster -f NPT_prod_pbc.xtc -s NPT_prod.tpr -method gromos -cutoff 0.12 -cl clusters.pdb -o clusters.xpm -dist distances.xvg -n index.ndx -dt 100 -b 25000 -e 200000`.

9.3 Molecular docking

Molecular Docking can be used to identify existing binding sites on the wild-type amyloid for the ligand of interest, and also to test the binding affinity of the ligand to the generated mutants.

Given the unknown nature of the binding site, blind docking will be conducted. This means that the docking algorithm will scan the entire protein structure to locate potential binding sites. The designated search space should be sufficiently broad to encompass all prospective binding sites, including interfaces between monomers. To mitigate the risk of biasing results towards a single conformation, the docking should be made in multiple runs using a variety of conformations obtained from the MD simulations, thereby facilitating the identification of a consensus binding site.

Multiple quality programs, free and paid, are available to perform Molecular Docking such as Autodock4 (Morris et al., 2009), GOLD (Jones, Willett, Glen, Leach, & Taylor, 1997), VINA (Trott and Olson, 2010) or webservers like SwissDock (Grosdidier, Zoete, & Michielin, 2011). The use of different programs may help avoiding false positives and negatives.

For simplicity, we describe our approach using SwissDock webserver which is based on the protein-ligand docking program EADock DSS and has demonstrated similar performances to Autodock4 and VINA.

9.3.1 Software requirements

A web browser and a software for drawing and editing molecular structures such as Avogadro (Hanwell et al., 2012) or UCSF Chimera (Pettersen et al., 2004) which combines well with SwissDock.

9.3.2 Protocol

For the molecular docking, the coordinates of the multiple conformations of the amyloid obtained from the MD simulations are needed in pdb format alongside a coordinates file with the ligand in mol2 format. The ligand can be drawn in a chemical editor program or can easily be retrieved in pubchem (https://pubchem.ncbi.nlm.nih.gov/).

Check the structure of the ligand and verify if all hydrogens are correctly added.

Go to http://www.swissdock.ch/docking.

On both Target selection and Ligand selection boxes, click on **Upload file** and upload the pdb file of the protein and the mol2 file of the ligand respectively.

Click on **Show extra parameters**. There you can define a specific search space or leave it blank for a blind docking. You can also define a radius around the ligand, inside which the conformations of the residues' side chains will be optimized.

Give a job name and optionally give an email to receive a message when the docking finishes.

When the job finishes, you obtain a table with the calculated binding affinities for each pose. This table can be downloaded as a.csv file. The poses can be downloaded as a zip folder. You may visualize the poses with any molecular visualization software.

The most promising complex(es) should undergo the MD simulation protocol detailed on Section 8.2 to verify their stability and confirm the suggested binding sites. The ligand should be parametrized following the typical ANTECHAMBER protocol of parametrization of non-protein molecules using the GAFF2 force field.

9.4 Design of the catalytic amyloid

Our approach involves integrating an enzyme active site into a region of the amyloid where the ligand exhibits inherent affinity. By doing so, we ensure that the newly introduced catalytic site does not compete with existing binding sites that may be more favorable.

Our enzyme of interest, along with the enzyme:substrate complex across various stages of the catalytic cycle, has been elucidated and documented through QM/MM calculations, providing a solid foundation for transferring the active site into the amyloid structure.

The strategy we follow includes aligning the amyloid binding site using a conformation coming from an MD simulation of the amyloid:ligand complex and the enzyme's active site using the coordinates of the ligand as reference. This facilitates the rational optimization of the mutations needed to graft the active site residues into the amyloid scaffold.

For the introduction of point mutations, we will use the PyMOL mutagenesis tool, which utilizes the Dunbrack's Rotamer Library to obtain feasible conformations for the newly incorporated residues. RosettaMatch (Richter, Leaver-Fay, Khare, Bjelic, & Baker, 2011) can be used as an automated alternative.

9.4.1 Software requirements

PyMOL.

9.4.2 Protocol

To align the structures, use the Pair Fitting Wizard of PyMOL: Switch the Mouse Mode into 3-Button Editing and launch Pair Fitting from the Wizard Menu; Left click on an atom of the ligand on the structure that will move and then the corresponding atom on the one that will serve as template; repeat this at least three times; Once the atoms are selected, left click on **Fit x pairs** to perform the superimposition; Left click **Done** to exit the Pair Fitting Wizard.

To introduce the mutations on the amyloid binding site, launch the Mutagenesis Wizard of PyMOL from the Wizard menu. Left click on the residue you wish to mutate. On the Mutagenesis menu, click on **No Mutation** and choose the residue you wish to introduce. PyMOL will then display alternative rotamers based on the Dunbrack's library of rotamers. You can display the various possible rotamers with the video-control buttons at the bottom right of the screen; Eventual steric clashes are graphically displayed and strain value is shown on the terminal. Click **Done** after finishing. Repeat the process to introduce additional mutations. The resulting mutated pdb file can then be saved.

Repeating the Molecular docking and MD simulation protocols with the mutated protein is essential to validate the engineered amyloid. Docking allows to confirm if the mutations did not disturb the binding of the ligand to the designed catalytic site, while MD simulations probe the stability of the amyloid:ligand complex and examine the frequence of catalytically favorable conformations by analyzing critical distances necessary for the reaction.

This stage may require several iterations to optimize design.

9.5 QM/MM simulations

QM/MM calculations provide detailed atomic-level understanding of the catalytic process taking place at the engineered active site. The energy profiles derived from these calculations help understand the catalytic capabilities of the designed catalytic site. Additionally, examining the electrostatic interactions of residues remote from the catalytic site and their influence on energy barriers can refine the amyloid for more effective catalysis. Modifying distant residues through point mutations might be necessary to create a favorable electrostatic environment for the reaction through stabilization of the rate-limiting transition state. Note however that *these are expensive calculations*. To reduce the number of calculations, we recommend to conduct these calculations on designs previously tested

experimentally with promising results. Programs such as Gaussian (Frisch, 2009), ORCA (Neese, Wennmohs, Becker, & Riplinger, 2020), and CP2K (Kühne et al., 2020) are amongst the widely utilized programs for these calculations.

Here we provide a simple protocol to conduct QM/MM calculations using ORCA, on a chosen single frame obtained from an MD simulation of the complex between the promising amyloid design and the ligand, in which the distances between the catalytic site and the ligand are favorable for catalysis. The input examples should work for most cases. QM/MM MD calculations may be preferred if there is enough computational power available.

9.5.1 Software requirements

ORCA 5.0.x series.

9.5.2 Protocol

9.5.2.1 System preparation

From an MD simulation of the complex between the most promising amyloid design and the ligand, extract, in pdb format, a frame in which the distances between the catalytic site residues and the ligand are favorable for catalysis. Remove bystander atoms like counter-ions, unnecessary monomers and water molecules. Maintain a 4Å thick layer of water molecules surrounding the system or add it later in LEaP.

Generate a topology file for the treated pdb file using LEaP, analogous to the protocol detailed in Section 8.2 but without adding a water box or counterions. Remember to include the parameters of the ligand.

Convert the prmtop file generated to an ORCA parameter file using the command: `ORCA -convff -AMBER amyloid.prmtop`.

To define the QM region, create an atom selection that includes the atoms to be added to the QM region and set the occupancy to 1. You can do that on VMD's tkConsole or manually edit the occupancy column on the pdb file. It is good practice to select as QM atoms: (1) all atoms of the ligand and of the catalytic residues; (2) the residues of the first shell of interactions with the ligand; (3) important interactions with the catalytic residues. When selecting QM atoms, avoid cutting polarized bonds such as peptide bonds, aromatic rings and high order bonds and salt bridges. The QM region should not be too big to avoid slow calculations. A size of 150 atoms is usually a good compromise between speed and accuracy.

Define the active region (the sphere of atoms around the QM layer that will be allowed to move freely). Usually, a 6Å radius around the QM atoms is an adequate choice. The active region is defined by setting the beta value to 1 while atoms with beta value of 0 will remain frozen during the calculations.

9.5.2.2 Characterization of the catalytic reaction

To characterize the catalytic reaction, perform the following steps: (1) initial geometry optimization; (2) linear transit scans of the reaction coordinates of each catalytic step followed by transition state optimizations; (3) Intrinsic Reaction Coordinate calculations to accurately find the minima associated with each transition state followed by geometry optimizations of the obtained minima, and (4) single point energy calculations on each stationary state. Input models for each type of calculation are below. The inputs can be run with following command: `orca filename.inp > filename.out`.

Geometry Optimization

```
! QMMM b3lyp D3 def2-SVP Opt VerySlowConv
%maxcore 2000
%pal
 nprocs 16
end
%mm
 LJCutOffInner 10.
 LJCutOffOuter 12.
 CoulombCutOff 12.
 Do_NB_For_Fixed_Fixed false
end
%qmmm
 Use_QM_InfoFromPDB true
 Use_Active_InfoFromPDB true
 ORCAFFFilename "amyloid.prms"
end
*pdbfile 0 1 amyloid.pdb # Charge and Multiplicity of the QM layer
```

Linear transit scan and transition state optimization

```
! QMMM b3lyp D3 def2-SVP scanTS VerySlowConv
%maxcore 2000
%pal
 nprocs 16
end
%geom
 Scan
  B 1 2 = 2.3, 1.0, 20 # index of atom 1;index of atom 2 = initial
distance, final distance, number of steps
 end
end
%mm
 LJCutOffInner 10.
 LJCutOffOuter 12.
 CoulombCutOff 12.
 Do_NB_For_Fixed_Fixed false
end
%qmmm
 Use_QM_InfoFromPDB true
 Use_Active_InfoFromPDB true
 ORCAFFFilename "amyloid.prms"
end
*pdbfile 0 1 amyloid_opt.pdb # Charge and Multiplicity of the QM layer
```

IRC calculation

```
! QMMM b3lyp D3 def2-SVP IRC VerySlowConv
%maxcore 6000
%pal
 nprocs 16
end
%mm
 LJCutOffInner 10.
 LJCutOffOuter 12.
 CoulombCutOff 12.
 Do_NB_For_Fixed_Fixed false
end
%qmmm
 Use_QM_InfoFromPDB true
 Use_Active_InfoFromPDB true
 ORCAFFFilename "amyloid.prms"
end
*pdbfile 0 1 amyloid_TS.pdb # Charge and Multiplicity of the QM layer
```

Single point energy calculation

```
! QMMM b3lyp D3 def2-TZVPP VerySlowConv
%maxcore 2000
%pal
 nprocs 16
end
%mm
 LJCutOffInner 10.
 LJCutOffOuter 12.
 CoulombCutOff 12.
 Do_NB_For_Fixed_Fixed false
end
%qmmm
 Use_QM_InfoFromPDB true
 Use_Active_InfoFromPDB true
 ORCAFFFilename "amyloid.prms"
end
*pdbfile 0 1 amyloid_R.pdb # Charge and Multiplicity of the QM layer
```

9.5.2.3 Transition-state dipole stabilization

Identify all charged and polar residues within a 10Å radius of the QM layer and generate single mutants in which you mutate each of these residues to alanine.

For each single mutant, edit the pdb files of the optimized reactants, transition state and products that constitute the rate-limiting step, by deleting the side chain of the residue to be mutated beyond the C_β atom and changing its name to ALA.

Repeat the LEaP protocol to generate a pdb and a topology file of the generated mutant.

Conduct single-point energy calculations on the reactants, transition state and products of the rate-limiting step using the corresponding mutated pdb files for each single mutant.

Compare the obtained energy barriers with one obtained with the original amyloid design. The mutants that lead to a decrease on the energy barrier indicate that the original residue on that position is destabilizing the transition state dipole. In contrast, if the mutation increases the barrier, the original residue was stabilizing the transition state dipole. In this way, one can carry out further optimizations to the designed catalytic amyloid.

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