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REVIEW ARTICLE

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Experimental methods for studying amyloid cross-interactions

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Abstract

Interactions between amyloid proteins represent the cornerstone of various pathogenic pathways, including prion conversion and co-development of distinct kinds of systemic amyloidosis. Various experimental methodologies provide insights into the effects of such cross-interactions on amyloid self-assembly, which range from acceleration to complete inhibition. Here, we present a comprehensive review of experimental methods most commonly used to study amyloid cross-interactions both in vitro and in vivo, such as fluorescence-based techniques, high-resolution imaging, and spectroscopic methods. Although each method provides distinct information on amyloid interactions, we highlight that no method can fully capture the complexity of this process. In order to achieve an exhaustive portrayal, it is necessary to employ a hybrid strategy combining different experimental techniques. A core set of fluorescence methods (e.g., thioflavin T) and high-resolution imaging techniques (e.g., atomic force microscopy or Cryo-EM) are required to verify the lack of self-assembly or alterations in fibril morphology. At the same time, immuno-electron microscopy, mass spectrometry, or solid-state NMR can confirm the presence of heterotypic fibrils.

KEYWORDS
cross-interactions, cross-seeding, fibril polymorphism, high-resolution microscopy, thioflavin T

1 | INTRODUCTION

Protein aggregation into toxic species is frequently linked to a variety of human diseases, collectively known as protein misfolding diseases (PMDs) or proteinopathies. To date, 42 human fibril-forming proteins (amyloids) have been implicated in different proteinopathies (Buxbaum et al., 2024). These disorders range from neurodegenerative diseases, such as amyloid-β (Aβ) aggregation in Alzheimer's disease (AD) and α-Synuclein (AαSyn) aggregation in Parkinson's disease (PD) (Goedert, 2001; Hardy & Selkoe, 2002; Lücking & Brice, 2000), to endocrine diseases, like the involvement of islet amyloid polypeptide (AIAPP) in type 2 diabetes (T2D) (Hull et al., 2004), and visual impairments, such as cataract caused by crystalline aggregation (Roskamp et al., 2020).

Although the aggregation of proteins into fibrillar structures is often associated with the development of proteinopathies, this phenomenon is also utilized by many biological systems for functional purposes (Otzen, 2010). These naturally occurring functional amyloids, found in a wide range of organisms from bacteria to humans, are involved in various functions, such as cell adhesion, biofilm formation, hormone storage, and antimicrobial defense (Romero & Kolter, 2014; Van Gerven et al., 2015). In humans, the premelanosome amyloid protein (PMEL17) functions prominently in pigment biosynthesis (Louros et al., 2016) and antimicrobial peptides produced by the innate immune system undergo amyloid-like aggregation, contributing to antibacterial defense by disrupting bacterial cell membranes (Bücker et al., 2022).

The term 'amyloid' initially used to describe extracellular deposits of abnormally folded protein aggregates, has broadened to encompass any peptide or protein that forms cross-β-sheet fibrils (Buxbaum et al., 2024). Regardless of their pathological or functional roles, amyloids undergo a fundamental process known as self-assembly (fibrillation), which enables proteins or peptides to spontaneously form highly ordered, multiscale fibrillar structures in various biological contexts (Dobson, 2003). This multi-stage phenomenon (Figure 1a,b) could involve both primary and secondary nucleation, during which protein monomers spontaneously aggregate into dimers, subsequently forming oligomers. These oligomers further polymerize into protofibrils and eventually mature amyloid fibrils, undergoing structural changes throughout the process (Cohen et al., 2011; Törnquist et al., 2018). Environmental factors could reverse this polymerization, with fibrils disassembling back into oligomers or monomers under specific conditions (Pálmadóttir et al., 2025).

The complexity of amyloid self-assembly is coupled with structural variability deriving from a phenomenon known as 'amyloid polymorphism' (Close et al., 2018). This variability is characterized by diverse fibril architectures, including twisted, helical, and rod-like structures, as well as multistranded fibril nodules and twisted ribbons (Annamalai et al., 2016). Polymorphism arises from variations in the number, orientation, or substructure of protofibrils (Fändrich et al., 2009; Fitzpatrick et al., 2013) and can also be influenced by experimental or physiological conditions, or minor structural modifications (Emendato et al., 2018; Zhou et al., 2021). Structurally distinct polymorphous fibrils can arise during self-assembly or due to cross-interactions between various amyloidogenic proteins (Zhang et al., 2015) making these fibrils even more challenging to study.

1.1 | Cross-interactions between amyloids

Self-assembly occurs through homogenous interactions among identical molecules and can be significantly modulated by environmental context. Such interactions could influence the speed of self-assembly and the conformation or composition of the resulting fibrils. One of the most distinguishable properties of amyloids is their ability to modulate the self-assembly of other fibril-forming proteins or peptides. This can happen through interactions between the same amyloids at different stages of assembly or through interactions between different amyloid proteins and can even lead to the development of hetero-fibrils composed of two or more amyloid types (Konstantoulea et al., 2022).

These interactions result in amyloid cross-seeding, cross-aggregation, or cross-amyloid inhibition (Ren et al., 2019). Cross-aggregation involves different monomeric proteins forming mixed aggregates with unique morphological characteristics and properties. In contrast, cross-seeding involves preformed seeds or critical oligomeric nuclei of one protein inducing or accelerating the fibrillation of another protein (Subedi et al., 2022). Cross-seeding can help overcome the energy barrier required for fibrillation when environmental or experimental conditions hinder aggregation (Ma & Nussinov, 2012).

The first data confirming the existence of molecular cross-talk between different amyloids was obtained by studying the molecular structure of protein deposits, a extracellular or intracellular accumulations of fibrils (Mena et al., 2009). The coexistence of heterologous protein aggregates, such as Aβ and AαSyn (Mandal et al., 2006), Aβ and ATau (Buggia-Prévot et al., 2014), Aβ and transthyretin (ATTR) (Li et al., 2013), AIAPP and insulin (Ivanova et al., 2021), has been found in amyloid deposits of patients simultaneously suffering from several PMDs such as AD, transmissible spongiform encephalopathies (TSEs), PD, and T2D (Ivanova et al., 2021; Luo et al., 2016a; Morales et al., 2013; Nonaka et al., 2018). High prion transmissibility and

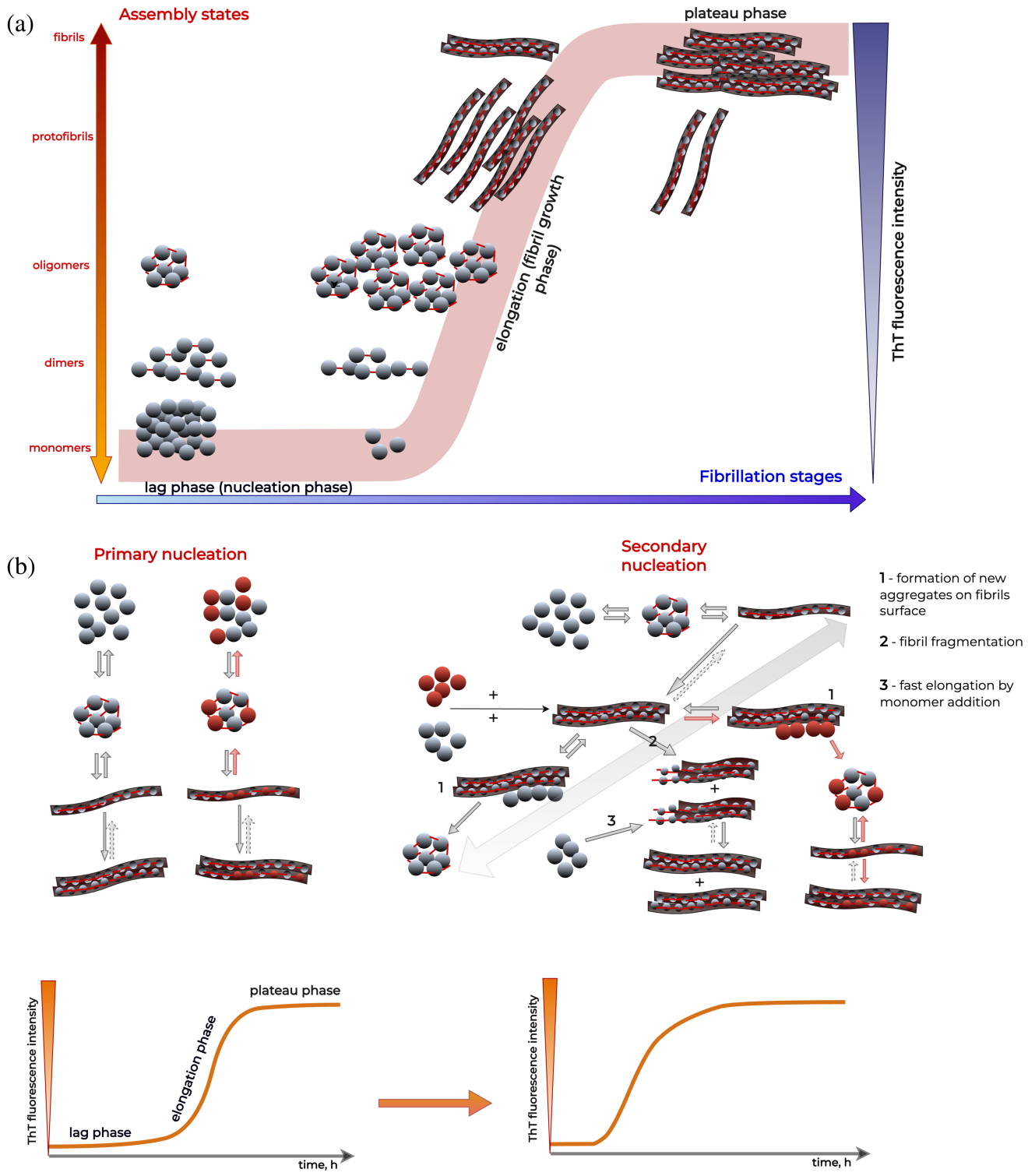

FIGURE 1 Overview of amyloid fibrillation and stability across assembly states. (a) Schematic representation of the three stages of amyloid fibrillation. The fibrillation process begins with the lag phase, where proteins exist in a dynamic equilibrium between soluble monomers and small aggregates, with minimal thioflavin T (ThT) fluorescence intensity observed. During the nucleation phase, these small aggregates cluster into a stable fibril nucleus, triggering a noticeable increase in ThT fluorescence as fibrils form. The elongation phase follows, characterized by the rapid addition of monomers to the growing fibrils and a corresponding sharp rise in ThT fluorescence intensity, indicating the formation of mature fibrils. Finally, in the plateau phase, fibril growth slows as the system approaches equilibrium, with ThT fluorescence intensity stabilizing as the concentration of remaining monomers diminishes. (b) The varying stability levels across distinct assembly states for heterotypic and homotypic amyloid fibrils. The assembly states range from stable, permanent interactions, marked as irreversible and characterized by strong intermolecular bonds between monomers, to transient strong interactions, which require specific environmental factors or conformational changes for bond dissociation. The transient weak interactions represent a reversible equilibrium, where bonds between monomers form and break spontaneously. Monomer populations of different proteins are shown in gray and red, with double arrows indicating the reversibility of the process. External factors that trigger dissociation are noted, with red lightning bolts denoting cases where a specific trigger is necessary for dissociation.

cross-species conversion are linked to cross-interaction and cross-seeding between different prion proteins (Luers et al., 2013; Soto, 2012; Vishveshwara & Liebman, 2009).

In vitro experiments also proved the capacity of some well-known amyloidogenic proteins to induce or accelerate the aggregation of other proteins in the process known as cross-seeding (Krebs et al., 2004). For instance, it has been shown that Aβ peptide, under specific conditions, seeds the polymerization process of AαSyn (Ono et al., 2012), ATau (Guo et al., 2006) or prion protein (APrp) (Morales et al., 2010).

Additionally, some functional amyloids rely on heterogeneous interactions as a basic principle (Zhou et al., 2012). In Gram-positive bacteria like *Staphylococcus aureus*, cross-interaction within phenol-soluble modulins (PSM) is crucial for biofilm formation, stabilization, increased virulence, and toxicity to non-self cells (Zaman & Andreasen, 2020). PSM fibril polymorphism is influenced by their propensity for self-assembly and cross-interaction within the family (Cracchiolo et al., 2022; Marinelli et al., 2016). Functional hetero-prion complexes are essential for biological functions, such as the localized synthesis of tubulin via co-aggregation of Sup35 protein with prion amyloids from the stress granules protein Tia1 (Li et al., 2014).

Strikingly, amyloid proteins of very different origins and entirely unrelated primary sequences have also been revealed to be capable of cross-interacting with each other. In particular, functional bacterial amyloids, like CsgA and PSMα were found to accelerate AαSyn aggregation in an experimental PD model in vitro (Werner et al., 2020), as well as in mice (Sampson et al., 2020). PSMα3 was also found to inhibit human insulin aggregation (Kalitnik et al., 2024). These findings might be explained by a cross-talk between bacterial functional amyloids and human pathogenic amyloids (Werner et al., 2020).

1.2 | Studies of amyloid interactions

Studying heterogeneous amyloid interactions is crucial for understanding the mechanisms underlying their self-assembly, particularly in the context of pathogenic amyloids, where aggregation can drive disease progression or inhibition. Gaining deeper insights into what accelerates or inhibits amyloid aggregation is of significant interest, as it could lead to new therapeutic interventions for amyloid-related diseases. Depending on the experimental approach, these interactions can be investigated using computational simulations, in vitro biochemical assays, or in vivo models, each offering distinct insights into the consequences of heterogeneous interactions.

Computational approaches, such as molecular threading, offer cost-effective strategies to explore aggregation mechanisms by, for example, the identification of interaction interfaces. These insights often guide the design of in vitro studies, which provide exact measurements of the kinetics of aggregation or the morphology of resulting fibrils. While this information is crucial for the mechanistic understanding of amyloid cross-interactions, in vivo experiments are vital for evaluating their physiological relevance and impact within the complexity of living systems.

However, because no single experimental method can fully capture the complexity of amyloid interactions, a combination of approaches is often necessary. It is also important to note that different techniques provide varying levels of directness in the evidence they offer, underscoring the need for a multifaceted analytical strategy. Some methods yield immediate, direct insights into amyloid structure and binding, while others require additional processing and interpretation, often involving additional calculations, probes, or markers.

2 | COMPUTIONAL STUDIES OF AMYLOID INTERACTIONS

Although computational methods have always played a significant role in the field of amyloid research (Santos et al., 2020), they are limited in analyzing amyloid interactions. Most studies in this area rely on molecular dynamics simulations with atomistic or coarse-grained force fields. Due to the high computational cost of such simulations, research tends to focus on interactions between shorter amyloid sequences, like AIAPP and Aβ (Baram et al., 2016) or prion protein peptides originating from different species (Wang & Hall, 2018).

While structure-based methods are central to modeling cross-interactions, sequence analysis complements them by revealing evolutionary and functional factors driving amyloid cross-talk. A motif-oriented study identified sequence constraints shaped by interactions between fungal prion amyloid motifs (Daskalov et al., 2015). Similarly, a phylogenetic approach explores the diversity and conserved motifs of CsgA homologs enabling cross-species interactions (Dueholm et al., 2012).

The release of AmyloGraph, the first database on amyloid interactions (Burdukiewicz et al., 2022), has enabled the development of specialized tools to predict how different amyloids might interact. PACT uses Modeler-based molecular threading to assess cross-interaction potential (Wojciechowski et al., 2023). AmyloComp not only evaluates the general cross-seeding potential, but also estimates the structural compatibility of sequences for hetero-fibril formation (Bondarev et al., 2024).

3 | FIBRILLATION-CENTRIC STUDIES OF AMYLOID INTERACTIONS

Since fibril-formation is a defining characteristic of amyloid proteins, most experimental methods investigate

the kinetics of self-assembly or the structural properties of resulting fibrils. This methodology expands naturally to studies of amyloid cross-interactions which similarly rely on tracking the impact of interaction on the self-assembly kinetics or morphology of resulting fibrils.

3.1 | Fluorescence-based techniques

Various fluorescence techniques have been widely used for detecting amyloid fibrils and studying the kinetics of protein aggregation (Xu et al., 2016). Their *modus operandi* involves the addition of an agent that alters its emission upon binding to amyloid fibrils. Thioflavin T (ThT), in particular, is a 'gold standard' fluorescence dye that has been used for monitoring amyloid fibril assembly for over fifty years (Gade Malmos et al., 2017). ThT binds to β-sheet-rich structures creating a characteristic blue shift in the emission spectrum (from approximately 510 nm in the free state to 480 nm) (Groenning, 2010; Sulatskaya et al., 2011).

In protein-only solutions, the interaction of ThT with mature amyloid fibrils is usually specific as fluorescence originates only from the dye bound to amyloid fibrils. Although ThT fluorescence analysis can be used to identify isolated amyloid fibrils or amyloids in tissue samples, this method is more commonly used for monitoring in vitro amyloid assembly over time (Groenning, 2010).

The kinetics of ThT fluorescence is typically described by a sigmoidal curve that reflects the conformational and morphological transformation of proteins during the fibrillation process, encompassing three different fibrillation stages (Figure 1a) (Lee et al., 2007). Thus, an increase in ThT fluorescence provides indirect evidence of protein aggregation. Furthermore, computational analysis of the time-dependent fluorescence change could elucidate the dominant nucleation mechanism. Such analysis could be conducted with Amylofit, which fits to the ThT kinetics mathematical models discriminating between six different mechanisms of aggregation covering all variants of fibrillation stages (Meisl et al., 2016).

Comparative analysis of ThT kinetics was vital in determining the sequence specificity of cross-seeding. The significant increase in ThT fluorescence when turkey lysozyme was seeded by hen lysozyme, compared to the absence of such a response when seeded by human α-lactalbumin, was sufficient evidence of the sequence-based constraints of cross-seeding (Krebs et al., 2004). This quality can also be exploited in the studies of cross-α-fibrils (Zheng et al., 2018). However, in this case, ThT seems to be less specific than for cross-β-fibrils when confronted with microscopy imaging (Tayeb-Fligelman et al., 2020).

Despite its advantages, ThT cannot reliably differentiate between mature amyloid and pre-fibrillar specimens. While ThT usually does not bind to amyloid precursors, it can detect certain dimers, trimers, or β-sheet-enriched oligomers (Carrotta et al., 2001), such as those of insulin (Grudzielanek et al., 2006), ATTR (Lindgren et al., 2005), and Aβ (Maezawa et al., 2008). This unspecific binding extends to globular proteins like acetylcholinesterase (De Ferrari et al., 2001) or albumin (Sen et al., 2009) but also to polysaccharides (Keliényi, 1967) or nucleic acids (Mohanty et al., 2013). Therefore, complementary biochemical and biophysical methods are essential to confirm ThT assay results and prevent misinterpretation due to staining of non-amyloid samples. Additionally, the emission intensity of ThT is not always linearly related to its binding, which is why equimolar concentrations are often recommended to avoid self-quenching and variability, as ThT binding reaches saturation at these concentrations (Lindberg et al., 2017). This underscores the need for careful calibration of ThT's emission response in quantitative in vitro studies.

Another common dye in amyloid aggregation studies is the sodium salt of benzidinediazo-bis-1-naphthylamino-4-sulfonic acid called Congo Red (CR). Initially used in histopathology to detect amyloid deposits (Yakupova et al., 2019), it has been employed to study in vitro amyloid fibrils. However, CR may affect the protein aggregation process by inhibiting oligomerization or disrupting existing aggregates; thus, while useful for identifying amyloids, CR staining alone is insufficient for studying protein cross-interactions.

In contrast to ThT and other dyes specific to amyloid fibrils in general, antibodies paired with fluorescence probes can selectively recognize distinct amyloid proteins. Traditional sequence-specific epitopes target defined linear regions, whereas structural epitopes additionally recognize conformational features associated with particular stages of fibrillation (Perchiacca et al., 2012). Due to their regional specificity, antibodies can elucidate the mechanisms of cross-interactions as in the case of AαSyn and lysozyme (Vaneyck et al., 2021). Additionally, conformationally sensitive antibodies map transitions through assembly states, expanding the understanding of the fibrillation kinetics as shown in the case of interactions between Aβ and TDP-43 (Shih et al., 2020).

3.2 | Imaging techniques

Morphological features of amyloid fibrils and early aggregates are often assessed using high-resolution imaging techniques. The most common methods are various forms of microscopy, including atomic force microscopy (AFM) (Adamcik et al., 2021) and electron microscopy (EM) (Gras et al., 2011).

3.2.1 | Atomic force microscopy

AFM imaging uses a scanning probe with a cantilever tipped to scan samples on a piezoelectric scanner. In

tapping mode, the cantilever performs oscillatory vibrations near its resonance frequency to 'tap' the sample, preventing damage (Butt et al., 2005). Oscillation amplitude variations are recorded as a three-dimensional topographic map of the sample, providing detailed morphological data on amyloid aggregates, including size, shape, height, width, and contour length, which are useful for tracking fibrillation kinetics and growth (Kellermayer et al., 2008). AFM also measures mechanical properties, such as stiffness, flexibility, and Young's modulus by applying force with an indenting probe (Ruggeri et al., 2019). Variations in these properties can indicate amyloid polymorphisms and differentiate fibrils from various protein samples (Kakinen et al., 2019).

More advanced AFM techniques provide interrelation between the structure and kinetic parameters; high-speed AFM is conducted in liquid environments to visualize single protein structures and dynamics in real time (Matveyenka et al., 2022; Watanabe-Nakayama, Nawa, et al., 2020). It is particularly useful for studying structural dynamics, fibrillation processes, cross-aggregation, protein aggregation inhibitors, and effects of surface properties on fibrillation (Adamcik & Mezzenga, 2012; Kellermayer et al., 2008; Pires et al., 2011; Watanabe-Nakayama, Sahoo, et al., 2020) of homologous (Kakinen et al., 2019; Liu et al., 2012) and heterologous fibrillation processes. Moreover, in cross-aggregation cases (Watanabe-Nakayama, Nawa, et al., 2020), high-speed AFM is used to search for inhibitors of protein aggregation (Kakinen et al., 2019) or explore effects of surface hydrophobicity (Keller et al., 2011) or surface changes (Moores et al., 2011; Wang et al., 2011) on the fibrillation mechanisms and kinetics.

However, the accuracy of AFM is its major limitation. The width of fibril images can vary based on tip geometry, necessitating calibration of tip dimensions for accurate measurements (Adamcik & Mezzenga, 2012). Samples may also interact with mica, altering their morphology (Bednarikova et al., 2020; McAllister et al., 2005; Zhu et al., 2002). Significant changes in fibril cross-sectional dimensions can indicate heterogeneous fibrils, but distinguishing these from homogeneous fibrils requires a detailed comparative analysis under controlled conditions (Heid et al., 2023; Seeliger et al., 2012). Therefore, AFM should be complemented by other methods, such as vibrational spectroscopy (Matveyenka et al., 2022; Rizevsky & Kurouski, 2020; Zhou et al., 2021) or electron microscopy.

AFM-IR is an advanced technique combining AFM with infrared (IR) spectroscopy, achieving a spatial resolution of 10 nm for nanoscale chemical and compositional imaging (Dos Santos et al., 2023; Xiao & Schultz, 2018). It probes protein aggregates by correlating morphological and IR absorption spectra of single fibrils (Otzen et al., 2021; Rizevsky & Kurouski, 2020; Waeytens et al., 2021). The technique allows for precise localization of IR signals and detailed observation of protein structures (Dos Santos et al., 2023; Otzen et al., 2021), providing an effective tool for studying protein oligomers, protofibrils, and fibrils (Banerjee & Ghosh, 2021; Waeytens et al., 2020, 2021; Xiao & Schultz, 2018), even in the presence of lipids (Rizevsky et al., 2022) and bacterial extracts (Otzen et al., 2021). Recent studies using AFM-IR have revealed distinct secondary structure distributions in hetero-fibrils formed by AαSyn and TDP-43 prion-like domains, highlighting heterotypic interactions and altered fibril morphology (Dhakal et al., 2021). Overall, the AFM-IR combines the advantages of AFM, including morphology mapping, IR absorption, and stiffness measurement, with unparalleled nanoscale chemical characterization. However, it requires complex instrumentation and can be prone to artifacts from sample preparation (Mathurin et al., 2022; Zhu et al., 2021).

3.2.2 | Electron microscopy

Another high-resolution technique commonly used for analyzing amyloid fibril morphology in vitro is EM (Gras et al., 2011). EM works by emitting electrons from a cathode, which are then accelerated, collimated, and focused before interacting with the sample. This interaction causes the electrons to lose kinetic energy, resulting in various effects such as absorption, reflection, or emission from the sample (Burghardt & Droleskey, 2006; Ryczkowski, 2012).

There are three types of EM, namely transmission electron microscopy (TEM), scanning electron microscopy (SEM), and cryo-electron microscopy (Cryo-EM). SEM is a straightforward, non-invasive technique commonly used to image surfaces and identify fibrils in tissue samples (Horvath et al., 2018; Thieu et al., 2022; Watanabe-Nakayama, Sahoo, et al., 2020), as well as track in vitro aggregation over time (Bai et al., 2008; Takai et al., 2014). For instance, SEM has effectively monitored fibril formation on silicon substrates without metal coating or staining due to attenuation effects (Thieu et al., 2022). However, compared to other methods, such as AFM or TEM, SEM provides lower resolution, contrast, and brightness, severely limiting its effectiveness for studying heteroaggregation or cross-interaction (Gras et al., 2011; Takai et al., 2014; Thieu et al., 2022), as well as details of the homoaggregation.

TEM creates images by passing electrons through a thin sample, allowing imaging of structures down to a few nanometers, including oligomers, protofibrils, and mature fibrils (Adil & Ramakrishnan, 2023; Burghardt & Droleskey, 2006). This technique provides both qualitative and quantitative data on fibril morphology, such as curvature, surface smoothness, and the periodicity of twists (Bruggink et al., 2012; Goldsbury et al., 1997; Gras et al., 2011; Periole et al., 2018). Like AFM, TEM

is effective for in vitro fibril formation monitoring over time (Shin et al., 2019).

A commonly used variation of TEM includes negative staining with a heavy metal stain. Uranyl acetate negative staining is highly effective for studying cross-seeding behaviors of amyloidogenic proteins over time (Liang et al., 2022), and polymorphological features of fibrils from heterogeneous self-assembly both in vitro (Ge et al., 2023; Ivanova et al., 2021; Liang et al., 2022; Lucas et al., 2022) and in vivo (Jiang et al., 2022). Enhanced contrast allows detailed visualization of fibril morphology, including general shape, size, length, diameter, and finer details such as protofibril width, crossover distance, and periodic twists (Liang et al., 2022; Lucas et al., 2022).

Studies using negative staining have revealed how different proteins influence fibril formation and morphology. For example, insulin rapidly fibrillates in the presence of various protein seeds, leading to aggregates with diverse morphologies (Akbarian et al., 2020). Hen egg-white lysozyme (HEWL) seeds significantly enhance BSA aggregation and alter its morphology (Nirwal et al., 2021). Additionally, TEM with negative staining has been used to investigate the effects of mutant Aβ isoforms on fibril assembly (Liang et al., 2022; Lucas et al., 2022), ATau protein interaction (Nizynski et al., 2018), and the impact of peptides such as AcPHF6 on Aβ fibrillation (Mohamed et al., 2018). Recent studies have also shown how severe acute respiratory syndrome coronavirus 2 spike amyloid seeds induce fibrillation in human prion protein and Aβ peptides (Larsson et al., 2023), and how casein and sericin interact with Aβ peptides to produce varied fibril morphologies (Ono et al., 2014).

Scanning transmission electron microscopy (STEM) combines transmission EM with scanning mode, offering a useful method for monitoring amyloid formation and assessing morphology, including heterogeneous aggregation. STEM uses a focused electron beam to scan the sample, with imaging resolution determined by the beam diameter (Sousa & Leapman, 2012). One of the most effective and frequent detectors in STEM is the annular dark-field detector system (ADF-STEM), which is particularly effective for measuring molecular masses of isolated macromolecules, for example determining mass per length of an amyloid fibril (Chen et al., 2009). Thanks to that, ADF-STEM was utilized to indicate different morphology of fibrils derived from seeding Aβ with seeds derived from two patients with different AD clinical history and neuropathology (Lu et al., 2013).

Immunolabeling approaches enable EM to determine the protein composition of assembled fibrils as it involves an additional step of labeling the material with specific antibodies, often conjugated with electron-dense particles such as gold nanoparticles. The high specificity of this method allows for the precise identification of hetero-fibrils such as ATau-AIAPP formed in vitro (Zhang et al., 2022). Moreover, the specificity of immunolabeling can be used to discriminate between co-aggregation and cross-seeding. For example, immunogold TEM showed that although Aβ can induce self-assembly of AαSyn, it is never incorporated into the core of fibrils (Vadukul et al., 2023). Similarly, the immunogold TEM indicated that seeds of lysozyme and insulin are incorporated into the mature fibrils of AαSyn (Yagi et al., 2005).

One of the main disadvantages of TEM, particularly when supplemented by negative staining, is the potential impact of heavy metal ions on fibril morphology or the fibrillation process itself (Berntsson et al., 2023; Thieu et al., 2022). Additionally, gold nanoparticles used in immuno-TEM can significantly accelerate or impede the fibrillation process or alter fibril morphology (Cabaleiro-Lago et al., 2008). Another drawback is the challenging sample preparation, as high-quality samples should have a thickness comparable to the mean free path of the electrons passing through them, not exceeding a few tens of nanometers (Eichberger et al., 2013). Despite these limitations, TEM remains one of the most effective tools for studying both the morphological polymorphism in amyloid cross-interaction and co-aggregation scenarios (Adil & Ramakrishnan, 2023).

Cryo-EM enables the in situ examination of hydrated samples that have been rapidly frozen in liquid ethane or propane, preserving their natural three-dimensional structure and protecting them from destruction (Grudzielanek et al., 2006; Zielinski et al., 2021). Cryo-EM does not require drying, staining, or surface deposition. It allows for the determination of near-atomic (less than 5 Å) protein structure in vitro and ex vivo through density map reconstruction using advanced computational methods (McGlinchey et al., 2021). To extract 3D morphological information, multiple images are needed (Cendrowska et al., 2020). Consequently, Cryo-EM facilitates the precise determination of parameters such as length, thickness, number of protofibrils, and twists in fibrils (Li et al., 2018). It also allows for the observation of dimers (Schmidt et al., 2016) and β-sheet-derived conformations that can form oligomers, protofibrils, and fibrils (Zielinski et al., 2021). Additionally, Cryo-EM enables the study of monomeric self-assembly, secondary nucleation, and surface-templated fibril growth during cross-interaction, which are challenging to identify using other microscopic methods (Gallardo et al., 2020). Importantly, Cryo-EM has shown that annexin A11 and TDP-43 form heteromeric amyloid fibrils during frontotemporal lobar degeneration with TDP-43 inclusions (Arseni et al., 2024).

The main challenge of Cryo-EM is the structural inhomogeneity of aggregates, which complicates the identification of specific polymorphs and makes the analysis of amorphous aggregates practically impossible (Yakupova et al., 2019; Zielinski et al., 2021). However, Cryo-EM offers advantages over other methods: unlike AFM,

samples do not need to be adsorbed onto a surface, and unlike TEM, the sample is not exposed to salt or low pH during the staining process (Gras et al., 2011).

3.2.3 | Super-resolution microscopy

Exceeding the diffraction limit, super-resolution microscopy provides a completely unique overview of molecular events. It is vital in the research on amyloids, where it enables a nanoscale visualization of mature fibrils (Bhuskute et al., 2024). Super-resolution microscopy combined with DNA-PAINT labeling allows for absolute quantification of events related to the seeding of AαSyn like the absolute number of aggregates inside and outside of cells, seeding probability, and the rate of secretion of fibrils (Sang et al., 2021).

3.3 | Spectroscopic methods

3.3.1 | Vibrational spectroscopic methods

Vibrational spectroscopic methods, including IR and Raman spectroscopy, analyze molecular interactions with IR radiation and light scattering. IR techniques, especially those combined with AFM or isotope labeling, are extensively used to study protein secondary structures, including amyloids, by examining amide bands (Wilkosz et al., 2020).

The Amide I band (1705–1595 cm^{-1}) is sensitive to protein secondary structures, with antiparallel β-sheets showing strong absorption near 1630 cm^{-1} and weaker near 1690 cm^{-1}. A decrease in the low-wavenumber band of β-sheets indicates structural changes or increased hydrogen bonding (Barth, 2007; Hauser, 2013). During aggregation, fibril formation is marked by a redshift of the Amide I band, increased intensity, and a significant decrease in full width at half maximum.

IR is advantageous for analyzing proteins in their native states across varying pH and ionic conditions with minimal sample preparation (Luers et al., 2013). However, the technique requires relatively high peptide and protein concentrations (Barth, 2007). A major drawback is the interference from water's bending mode around 1635 cm^{-1}, which overlaps with the Amide I band's α-helix and random coil components (Fellows et al., 2020). While IR spectroscopy effectively monitors structural transitions from native to misfolded and fibrillar forms, revealing detailed secondary structures throughout aggregation, it does not directly confirm cross-interactions and is primarily used to characterize fibrils (Dhakal et al., 2021; Surmacz-Chwedoruk et al., 2012; Ziaunys et al., 2023), including the lack of fibrillation resulting from cross-interactions (Kalitnik et al., 2024).

Vibrational circular dichroism (VCD) is a powerful spectroscopic technique that extends conventional circular dichroism (CD) analysis into the infrared region, providing detailed insights into the chiral centers and molecular interactions of amyloid fibrils (Measey & Schweitzer-Stenner, 2011). Unlike CD, which primarily detects changes in secondary structure (Kardos et al., 2025; Vadukul et al., 2019), VCD offers a more refined view of fibril morphology, amyloid polymorphisms, and intermolecular interactions, making it particularly valuable for studying amyloid–amyloid interactions (Krupová et al., 2021). Given that amyloid fibrils exhibit distinct chiral properties due to their highly ordered β-sheet arrangements, VCD can detect subtle alterations in chirality that arise during co-assembly or cross-interactions of different amyloid species. These changes in vibrational optical activity can provide valuable information on molecular rearrangements occurring in heterotypic fibril formation and structural remodeling (Kurouski et al., 2014). When used alongside other biophysical methods, VCD contributes to a more comprehensive understanding of amyloid aggregation mechanisms and cross-interactions.

3.3.2 | Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy provides detailed information about protein structure and dynamics at the molecular level. The sample is placed in a strong magnetic field and subjected to radio-frequency pulses, causing atomic nuclei spins to align parallel or antiparallel to the magnetic field lines. Absorption of electromagnetic radiation alters the spin states, and the frequencies of the absorbed radiation are recorded to produce a spectrum, revealing insights into the supramolecular structure of proteins in the sample (Gerothanassis et al., 2002).

NMR spectroscopy is effective for studying amyloid aggregation at all of its stages, from conformational transitions of monomers through oligomer formation to the structural characterization of mature fibrils (Karamanos et al., 2015). At the monomer level, NMR can be used to investigate the structure of individual monomers as well as conformational changes induced by cross-interactions. For instance, the two-dimensional 1H-15N Heteronuclear Single Quantum Correlation NMR has been shown to detect changes in the chemical shifts of residues 15–17 and 34–36 of Aβ upon insulin addition, suggesting that these regions interact with insulin monomers and inhibit its aggregation (Luo et al., 2016b). A similar study examined interactions between ATTR and its mutants with Aβ, identifying crucial amino acid residues for these interactions (Li et al., 2013). Moreover, sequential determinants crucial for the acceleration of AαSyn aggregation stimulated by TDP-43 Prion-like domains were also detected (Dhakal et al., 2021).

Since amyloid fibrils are typically noncrystalline, insoluble aggregates, solid-state NMR (ssNMR) is frequently used to study their architecture and structural

dynamics (Tycko, 2011). The principles of ssNMR are similar to those of liquid-state NMR spectroscopy and are based on the effects of magnetic fields and radiofrequency pulses on nuclear spin interactions (Reif et al., 2021). When combined with magic angle spinning (MAS), which eliminates anisotropic interactions and improves technique sensitivity, larger linewidths in ssNMR spectra enable the collection of data on the molecular structure, conformation, and dynamics of the studied material (Leskes, 2018; Meier et al., 2017). ssNMR of amyloid fibrils yields high-resolution quantitative data on their supramolecular structure and peptide conformations (Loquet et al., 2018; Tycko, 2011).

ssNMR has been used to determine the structure of fibrils formed by RIPK1 and RIPK3 proteins, as well as hetero-aggregates formed by both proteins together. The observed consistency of chemical shifts indicated that the core region is very stable, with the most variability occurring in the flanking regions. This stability allowed for the reconstruction of the molecular structure using computational methods (Mompeán et al., 2018). A similar approach was used to confirm the formation of hetero-fibrils by Aβ40 and Aβ42 variants and to elucidate the molecular structure of these aggregates (Cerofolini et al., 2020). An even more striking example employed ssNMR to showcase the templating of Aβ40 and Aβ42 fibrils by seeds obtained from patients with different clinical subtypes of AD (Qiang et al., 2017).

The main limitations of NMR spectroscopy are the high cost of equipment and its maintenance (Tampieri et al., 2021). Achieving a good signal-to-noise ratio often necessitates high concentrations of isotopically labeled proteins, which can increase aggregation (Karamanos et al., 2015; Martial et al., 2018). Additionally, since NMR-active nuclei other than hydrogen are scarce in biological samples, isotope labeling is generally required. Another challenge in NMR spectroscopy is signal broadening that occurs with larger test objects. Signals are influenced by various interactions around the nucleus, such as dipolar interactions, which depend on the molecule's orientation relative to the magnetic field. In solution, rapid molecular motion averages out these effects, creating an isotropic system (Marion, 2013). However, as the size of the molecules increases and their motion slows, anisotropic effects become more pronounced, resulting in weak and broad signals. This limits the size of macromolecules that can be effectively studied in solution, although this issue can be partially mitigated using ssNMR (Karamanos et al., 2015).

3.3.3 | Mass spectrometry

Mass spectrometry (MS) has become a powerful analytical technique for detecting, structurally characterizing, and studying the folding and aggregation of proteins and peptides. It enables time-resolved analysis of dynamic processes with structural resolution (Chen & Urban, 2013; Pukala, 2023; Ramesh, 2019). One of its main advantages is the ability to analyze molecules in heterogeneous mixtures without ensemble averaging, maintaining equilibrium conditions throughout the process. For instance, if aggregated proteins or oligomers are enzymatically digested before MS analysis, the resulting peptides can be separated based on their *m*/*z* ratios, allowing for protein identification from biological samples (Ramesh, 2019; Wagner & Gross, 2024).

Large biomolecules, such as proteins, require soft ionization techniques to minimize fragmentation (Banerjee & Mazumdar, 2012; Bronsoms & Trejo, 2015). Matrix-assisted laser desorption/ionization (MALDI), usually coupled with a time-of-flight mass spectrometer (TOF), applies a laser energy-absorbing matrix to generate gaseous ions of peptides and proteins, predominantly in the single charge state. This technique provides qualitative data on the oligomeric composition based on *m*/*z* values (Bronsoms & Trejo, 2015). MALDI-TOF MS has been used to detect various sizes of oligomers and isomerized variants of the Aβ peptide (Pekov et al., 2018; Wang et al., 2018), as well as alterations in its isoform pattern under secretase inhibitors (Portelius et al., 2011).

Native electrospray ionization (ESI) ESI-MS, with its mild ionization process using volatile ammonium-based buffers, preserves interactions between macromolecules by maintaining non-covalent bindings. This technique facilitates the direct detection and distinction of various soluble oligomers and intact fibril assemblies (Hu & Zheng, 2020; Tamara et al., 2022). Ion-mobility mass spectrometry (IM-MS) provides information on stoichiometry, composition, protein interactions, and topology of protein complexes, capturing the repertoire of conformational states adopted by protein assemblies (Ben-Nissan & Sharon, 2018). It also allows for continuous monitoring of oligomer formation and evaluation of the structure and self-assembly mechanisms of higher-order amyloid aggregates (Woods et al., 2013).

ESI-IMS-MS can be employed to investigate the interactions between various amyloids and characterize the consequent heterogeneous aggregates. For instance, this technique allowed the identification of hetero-fibrils formed after co-incubation of human and rat AIAPP in vitro (Young et al., 2014). Furthermore, ESI-IMS-MS was also applied to study the interaction between AIAPP and Aβ40 in vitro at an equivalent molar ratio. MS spectra analysis revealed that AIAPP and Aβ40 monomers combined randomly to form heterogeneous assemblies. Notably, AIAPP accelerated Aβ40, with the kinetics of heterogeneous polymers resembling those of the more aggregation-prone AIAPP and significantly faster than Aβ40 alone (Young et al., 2015). Therefore, MS has proven to be a versatile technique, offering complementary insights into the process of amyloid fibril formation through the integration of various experimental approaches (Pukala, 2023). However, limitations include high costs, the complexity of the equipment, and challenges related to

sample preparation (Hu & Zheng, 2020). For native MS for detecting intact protein assemblies, multidimensional separation is essential since oligomeric complexes can overlap at the same *m*/*z*, or a single species may exist in different conformational states (Tamara et al., 2022).

3.4 | Immunoprecipitation-based approaches

Co-immunoprecipitation (Co-IP) is a versatile technique used to detect physical interactions between proteins by utilizing antibodies targeting a protein participating in a protein complex such as mature fibril. A subsequent analysis of the isolated complex (e.g., MS) could be added to further characterize the exact fibril composition. This method is particularly useful when analyzing amyloid interactions within in vivo systems where direct observation is not possible.

Tissue samples are among the most commonly used materials for Co-IP, as they preserve native protein interactions occurring in physiological conditions. For example, Co-IP was used to showcase that AIAPP co-aggregates with ATau in human AD hippocamp (Zhang et al., 2022). Moreover, Co-IP indicated that in samples of brain tissue of AD patients, yeast prion Ure2p co-localized with human ATau (Meng et al., 2023).

4 | PHENOTYPE-BASED STUDIES OF AMYLOID INTERACTIONS

Understanding the molecular mechanisms of amyloid cross-interactions in vitro does not always reflect their functional consequences in living organisms. Therefore, phenotype-based, in vivo studies reveal within a cellular or organismal context the impact of these interactions that would not be visible in more reductionist systems. These approaches allow researchers to link molecular-level knowledge of these interactions with observable phenotypic outcomes, such as toxicity or disease progression.

Model organisms provide a useful resource for studying the phenotypes of progressing amyloid diseases (Buxbaum, 2009). Especially, microinjections of amyloids into model organisms could lead to the progression of associated proteinopathies. FapC seeds injected into zebrafish promoted Aβ aggregation, evidenced by CR-stained brain tissues and impaired motor functions (Javed et al., 2020). Injections of ATau-AIAPP fibrils into the hippocampus of transgenic mice showcased that previously shown in vitro seeding activity translates to the initiation of tauopathy (Zhang et al., 2022). Injections of ATau seeding with yeast protein Ure2p into the brains of transgenic mice led to much more extensive cognitive impairment than pure Tau fibrils (Meng et al., 2023).

Amyloid proteins fused with tags facilitate the visualization of their aggregation behavior within cells. Tagging prions with fluorescent markers enabled investigation of their colocalization and templating of fibril morphology in yeast (Du & Li, 2014).

The expression of selectively truncated protein fragments allows for characterizing their intrinsic amyloid-forming potential. In studying amyloid cross-interactions, these fragments can function as seeds for wild-type amyloid proteins or proteins that do not typically exhibit amyloid-like behavior. In fungi *Chaetomium globosum*, truncated HeLo-like domain protein (HELLP) constructs with prion-like properties were used to seed the full-length protein, leading to cell death (Daskalov et al., 2016). This

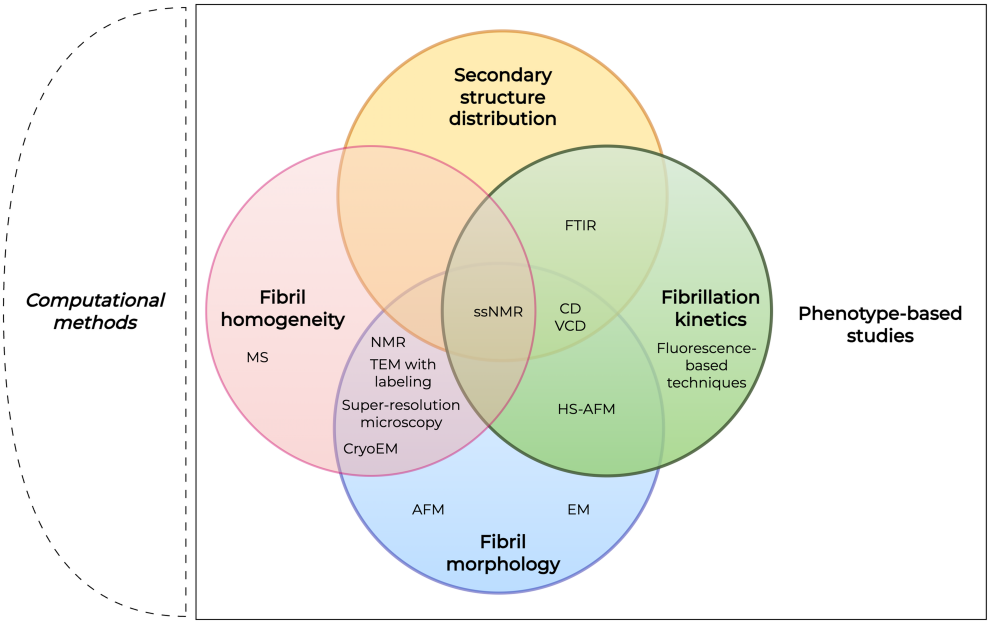

FIGURE 2 Diverse approaches and their relevance to amyloid aggregation analysis. Relevance of diverse experimental methods to four crucial aspects of amyloid aggregation: Fibrillation kinetics, secondary structure, fibril morphology, and fibril homogeneity. Each method is positioned according to the specific aspect(s) it can effectively examine, highlighting the complementary nature of these techniques. This visual representation underscores the importance of integrating multiple approaches to comprehensively understand amyloid aggregation and cross-interactions.

behavior mimics the function of the HET-S protein, a prion responsible for inducing cell death in other species of fungi, *Podospora anserina* (Balguerie et al., 2003).

Besides elucidating the function of novel amyloid proteins, constructs could be instrumental in the search for therapies against amyloid-related diseases. Peptides designed to mimic the Aβ amyloid core were found to alleviate the effects of long-term potentiation impairment on the ex vivo brain samples by inhibiting self-assembly of IAPP and Aβ42 in addition to suppressing their mutual cross-seeding (Taş et al., 2022).

Genetic engineering is essential for elucidating the specific sequence elements required for the amyloidogenic properties of a protein in vivo. In the case of PGRP-LE, a protein of *Drosophila melanogaster* involved in its humoral response, it enabled the identification of subsequences necessary for fibril formation, as well as those that inhibit this process. Only constructs of PGRP-LE with amyloid propensity were able to interact with another amyloid protein, Imd, leading to cell death (Kleino et al., 2017).

5 | CONCLUSIONS

Among the plethora of existing methods for studying amyloid self-assembly, only a fraction is suitable to be adapted to provide insight into the interactions between different amyloid proteins during the aggregation. The inference on the cross-interactions is based on either indirect information resulting from altered kinetics of aggregation, secondary structure, or the fibril morphology, or direct information on the fibril homogeneity (the presence of heterogeneous fibrils) (Figure 2).

ThT and other fluorescence-based methods can confirm cross-interactions between two amyloidogenic proteins by detecting altered aggregation kinetics. These changes are reflected not only in different aggregation rates but also in the altered length of the lag phase or different maximum fluorescence. Due to its relatively high accessibility, this method often becomes the first choice to verify the putative impact of cross-interaction.

TEM and AFM are examples of high-resolution imaging techniques for analyzing fibril morphology and polymorphism. These methods are appropriate for investigating amyloid formation throughout time and distinguishing and characterizing various assembly states such as oligomers, protofibrils, and full amyloid aggregates. TEM can also be extended to almost provide the direct confirmation of hetero-aggregates in conjunction with negative staining. However, immuno-TEM is the critical high-resolution imaging approach for displaying direct evidence of the presence of heterogeneous fibrils and characterizing their architecture. On the other hand, super-resolution microscopy allows scrutiny of the fibrils on an unprecedented scale, including the qualitative analysis of aggregates.

Vibrational spectroscopy, the most universal approach to secondary structure analysis, provides unique insights into the β-sheet types and fractional distribution associated with specific fibril morphologies, which are likely affected by cross-interactions. However, similarly to the fluorescence-based counterparts, these methods should be complemented with high-resolution imaging techniques, like AFM-IR, as they cannot provide direct evidence of interactions.

A common challenge in amyloid research is detecting and distinguishing multiple amyloidogenic proteins within the same fibril. Co-IP selectively pulls down amyloid fibrils, enabling subsequent interaction analysis with more discriminative methods, including MS and NMR. These approaches can also provide direct confirmation of the cross-interaction of two separate amyloids, as well as substantial information on the hetero-aggregate supramolecular structure, including the configuration of the amino acids that constitute initial proteins. Furthermore, these methods can be utilized to monitor the fibrillation process over time. Still, their demanding infrastructural requirements and harder interpretability make them less common in studies on amyloid cross-interactions.

Each of the described methods provides a partial insight into amyloid cross-interactions. On their own, they remain informative but still very limited in grasping the intrinsic complexity of this phenomenon. Thus, the only recommended approach is to combine indirect and direct evidence to create a more complete outlook of this process. This set of methods depends on the final result of the interaction. The minimum set of experiments necessary to confirm the lack of self-assembly or measure the alteration of fibril morphology consists of kinetics- and secondary structure-monitoring methods, as well as high-resolution imaging techniques. However, to confirm the presence of hetero-aggregates, it is necessary to additionally employ immuno-TEM, MS, or ssNMR. Moreover, phenotype-based studies are obligatory to fully investigate the impact of amyloid cross-interaction on the living organism.

AUTHOR CONTRIBUTIONS

Aleksandra Kalitnik: Conceptualization; writing – original draft; writing – review and editing; methodology; investigation; visualization; data curation. **Anna Lassota:** Conceptualization; investigation; writing – original draft; writing – review and editing; methodology. **Oliwia Polańska:** Writing – review and editing; investigation. **Marlena Gąsior-Głogowska:** Writing – review and editing; investigation. **Monika Szefczyk:** Writing – review and editing; investigation. **Agnieszka Barbach:** Writing – review and editing; investigation. **Jarosław Chilimoniuk:** Writing – review and editing; investigation. **Izabela Jęśkowiak-Kossakowska:** Writing – review and editing; investigation. **Alicja W. Wojciechowska:** Writing – review and editing; investigation. **Jakub W. Wojciechowski:**

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CONFLICT OF INTEREST STATEMENT

The authors report there are no competing interests to declare.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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