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REVIEW ARTICLE

Biological and methodological complexities of beta-amyloid peptide: Implications for Alzheimer's disease research

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Abstract

Although controversial, the amyloid cascade hypothesis remains central to the Alzheimer's disease (AD) field and posits amyloid-beta (Aβ) as the central factor initiating disease onset. In recent years, there has been an increase in emphasis on studying the role of low molecular weight aggregates, such as oligomers, which are suggested to be more neurotoxic than fibrillary Aβ. Other Aβ isoforms, such as truncated Aβ, have also been implicated in disease. However, developing a clear understanding of AD pathogenesis has been hampered by the complexity of Aβ biochemistry in vitro and in vivo. This review explores factors contributing to the lack of consistency in experimental approaches taken to model Aβ aggregation and toxicity and provides an overview of the different techniques available to analyse Aβ, such as electron and atomic force microscopy, nuclear magnetic resonance spectroscopy, dye-based assays, size exclusion chromatography, mass spectrometry and SDS-PAGE. The review also explores how different types of Aβ can influence Aβ aggregation and toxicity, leading to variation in experimental outcomes, further highlighting the need for standardisation in Aβ preparations and methods used in current research.

KEYWORDS
Alzheimer's, Alzheimer's disease, amyloid beta, Aβ, fibrils, oligomers, SDS-PAGE, sodium dodecyl Sulphate-polyacrylamide gel electrophoresis

1 | INTRODUCTION

1.1 | Alzheimer's disease

Alzheimer's disease (AD) is the most prevalent of neurodegenerative diseases. Clinically, AD is associated with memory impairments, as well as executive dysfunction, language problems and difficulties in carrying out daily activities. However, symptoms can vary between affected individuals (Grontvedt et al., 2018). Neuropathologically, AD is characterised by the presence of intraneuronal neurofibrillary tangles composed of hyperphosphorylated tau protein, and extracellular amyloid plaques composed mainly of fibrillary amyloid-β

Abbreviations: AD, Alzheimer's disease; ADDLs, amyloid β-derived diffusible ligands; AFM, atomic force microscopy; APP, amyloid precursor protein; Aβ, amyloid-β; BACE-1, beta-secretase 1; CD, circular dichroism; CSF, cerebrospinal fluid; CSI, cold spray ionisation; CTF, C-terminal fragments; DMSO, dimethyl sulfoxide; EM, electron microscopy; ESI, electrospray ionisation; fAD, familial Alzheimer's disease; HFIP, hexafluoro-2-propanol; IM, ion mobility; m/z, mass to charge ratio; MALDI, matrix-assisted laser-desorption ionisation; MS, mass spectrometry; NMR, nuclear magnetic resonance; PICUP, photo-induced cross-linking of unmodified proteins; sAD, sporadic Alzheimer's disease; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; ssNMR, solid-state NMR; TEM, transmission electron microscopy; ThT, Thioflavin T; TOF, time of flight.

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(Aβ), which, in the case of neuritic plaques, are surrounded by dystrophic neurites containing tau aggregates (Aleksis et al., 2017; Alzheimer et al., 1995).

Sporadic cases of AD (sAD) typically have a later onset of disease (above the age of 65), whilst familial AD (fAD) cases may occur earlier in life (van der Flier et al., 2011). fAD can be caused by autosomal dominant mutations in the *APP* gene (Goate et al., 1991), *PSEN1* (Sherrington et al., 1995), or *PSEN2* (Levy-Lahad et al., 1995) genes. *APP* encodes the amyloid precursor protein, whilst *PSEN1/2* encode presenilin proteins (van der Flier et al., 2011). APP is a protein from which Aβ can be cleaved. Presenilin proteins possess γ-secretase activity and are involved in generation of Aβ peptide from APP (De Strooper et al., 2012).

sAD is not associated with any specific genetic mutations (Isik, 2010), but there are genetic risk factors. The largest of these is *APOE*, specifically possession of the ε4 allele (Liu et al., 2013; Tzioras et al., 2019). The role of ApoE4 in AD is not yet fully understood, but it may be important in Aβ processing (Isik, 2010). Variants in *TREM2* have also been associated with AD pathogenesis, and these may affect inflammatory processes in the brain (Guerreiro et al., 2013; Jonsson et al., 2013). Recently, it has been found that TREM2 defects adversely affect microglia in AD, exacerbating the disease, as seen in TREM2 mice knockout experiments (Griciuc et al., 2019). Expression, and subsequent engagement, of the common variant of TREM2 could be used as a potential therapeutic option in AD (Wang et al., 2020). Polygenic influences are important in sporadic late onset AD. Large Genome Wide Association Studies (GWAS) have identified rare polymorphisms with smaller risk effects. Analysis of these variants and the pathways in which they are involved are providing additional insights into mechanisms in AD, but also include APP metabolism and Aβ formation (reviewed in (Bertram & Tanzi, 2020)).

1.2 | Amyloid-beta

Amyloid-beta (Aβ) monomers are small peptides of ~4 kDa (Glenner & Wong, 1984; Murphy & LeVine, 2010). Aβ is highly conserved in vertebrates and may have an important biological function. It has been suggested that native Aβ could have antimicrobial, antifungal and antiviral properties (Bourgade et al., 2015; Soscia et al., 2010). There is some statistical evidence that Aβ may also be involved in tumour suppression, maintenance of blood brain barrier and regulating synaptic functions (Brothers et al., 2018; Driver et al., 2012). However, the exact physiological function of Aβ remains to be elucidated, and most reports exploring Aβ function focus on its adverse effects in disease.

Aβ is the primary component of extracellular plaques (both diffuse and neuritic) that deposit throughout the medial temporal lobe and cortex in AD brain progressing into deep grey nuclei, brainstem and finally cerebellum at later phases of the neuropathology (Thal et al., 2002). Aβ deposition is accompanied by serum amyloid P, a universal component of human amyloid deposits, which may have a role in aggregation and plaque formation (Hamazaki, 1995; Kalaria et al., 1991), and which may itself have an association with dementia (Ellmerich et al., 2021). Aβ has been the focus of intense research efforts since the proposal of the amyloid cascade hypothesis. This hypothesis first posited that aggregation of Aβ in the brain is a vital first step in a cascade of events leading to the development of AD (Hardy & Higgins, 1992; Selkoe & Hardy, 2016).

The *APP* gene located on chromosome 21 encodes a transmembrane protein called amyloid precursor protein (APP). Proteolytic processing of APP leads to the generation of Aβ peptide. Non-amyloidogenic APP cleavage occurs by the enzymatic action of α-secretase, which prevents generation of Aβ, as the enzyme cleavage site is located within the Aβ sequence. Amyloidogenic processing of APP occurs via sequential cleavages by β- and γ-secretase enzymes (O'Brien & Wong, 2011; Sharma et al., 2017; Thinakaran & Koo, 2008), with β-secretase being the first and rate-limiting step. This liberates soluble APPβ and a β-C-terminal fragment (β-CTF) (Chow et al., 2010; O'Brien & Wong, 2011; Zhang et al., 2011). β-CTF is then further cleaved by γ- secretase to generate Aβ.

The proteolytic activity of γ-secretase is non-specific and therefore produces Aβ isoforms of varying sizes at the N- and C-termini of the Aβ peptide. The most commonly investigated isoforms are $Aβ_{1-40}$ and $Aβ_{1-42}$ (Chow et al., 2010; Edbauer et al., 2003), but numerous other isoforms exist including N-terminally truncated isoforms (for example $Aβ_{4-42}$, $Aβ_{5-42}$, $Aβ_{3-40}$), as well as variants with differing C-termini ($Aβ_{1-37}$, $Aβ_{1-38}$ and $Aβ_{1-39}$). $Aβ_{1-42}$ is the main component of plaques in AD and displays a high propensity to form fibrils in vitro; however, other isoforms have been found in plaques as well (Jarrett et al., 1993; Lührs et al., 2005). Post-translationally modified forms of Aβ have also been observed and investigated and will be discussed further in the review.

Aβ heterogeneity has complicated the field of AD research. Various sequence isoforms of Aβ have differing propensities to aggregate and induce toxicity, and post-translational modifications of the peptide, as well as conformational diversity of the different forms of Aβ, also affect these properties. Given the complexity in Aβ isoforms and their various aggregation products, there is a requirement for careful experimental design to ensure that the effects of Aβ in AD are reliably modelled.

The involvement of Aβ in AD pathogenesis is supported by studies of known causative mutations for fAD: *APP*, *PSEN1* and *PSEN2* (Bekris et al., 2010). Mutations in the *APP* gene may lead to altered Aβ metabolism in the brain. APP genetic variants, for example D678H (Taiwanese), E693G (Arctic) and E682K (Leuven), have been associated with increased Aβ production, increased Aβ oligomerisation and increased formation of Aβ fibrils, which are all associated with AD pathogenesis (Chen et al., 2012; Di Fede et al., 2009; Nilsberth et al., 2001; Zhou et al., 2011). In contrast, other APP variants, such as A673T (Icelandic), reduce Aβ production and may be protective against AD (Jonsson et al., 2013). Mutations in *PSEN1* and *PSEN2* genes result in abnormal production of Aβ (Liu et al., 2013). For example, loss of-function mutations in the *PSEN*

genes may lead to an increased in vitro and in vivo production of $A\beta_{1\text{-}42}$, which is thought to be a more toxic Aβ variant (Cacquevel et al., 2012; Potter et al., 2013). This implicates Aβ as a contributing factor to AD pathogenesis.

1.3 | Nucleation polymerisation

Aggregation of Aβ into mature fibrils is a complex process, which involves Aβ monomers as the starting point (Bartolini et al., 2011). Nucleation, also called nucleation polymerisation, is a mechanism of ordered polymerisation of proteins. Aβ aggregation is kinetically dependent on nucleation events, which includes primary and secondary nucleation. Primary nucleation refers to initial events where peptide monomers associate into nucleation 'seeds'. This is a rate-limiting, kinetically unfavourable process, resulting in a long lag phase (Ghosh et al., 2016; Xue et al., 2008). However, once the nuclei are formed, addition of further monomers is more favourable through an elongation process. Spontaneous association of monomers into oligomers or fibrils is determined by their stability, which can be dependent on the environment the peptide is found in Nichols et al., 2005; Tornquist et al., 2018. Despite evidence that Aβ monomers may have neuroprotective functions in the brain (Giuffrida et al., 2009), they are critical for the formation of toxic oligomers, intermediate protofibrils and mature fibrils which are the main component of plaques in AD brains (Jan et al., 2011; Linse, 2017).

Primary nucleation causes Aβ elongation into fibrils, which eventually leads to the formation of a critical mass of fibrils capable of catalysing the formation of new fibrils by the process of autocatalysis. This creates a feedback loop, which can take the form of simple processes such as fibril branching or fragmentation, but for Aβ, it is dominated by secondary nucleation (Cohen et al., 2013; Linse, 2017). In this process, the formation of new nuclei is catalysed by the surface of existing fibrils; this leads to an exponential growth of Aβ aggregates (Cohen et al., 2013; Jarrett et al., 1993). Secondary nucleation overrides primary nucleation and leads to rapid Aβ polymerisation. The aggregation process is dependent on the relative concentration of both oligomers and fibrils, and hence, the system acts in a positive feedback loop, with secondary nucleation taking place in an auto-catalytic cycle (Figure 1) (Cohen et al., 2013). The exponential growth takes place, because an increased number of fibrils leads to more fibril availability, which catalyses the formation of new nuclei. Oligomers formed during secondary nucleation are thought to be the most neurotoxic species (Cohen et al., 2013) and can exist in an equilibrium with Aβ fibrils comprising plaques in AD brains (Yang et al., 2017). The aggregation process continues, until an equilibrium resulting in a fibril growth plateau is reached; this takes place when all free monomers have been converted into Aβ fibrils.

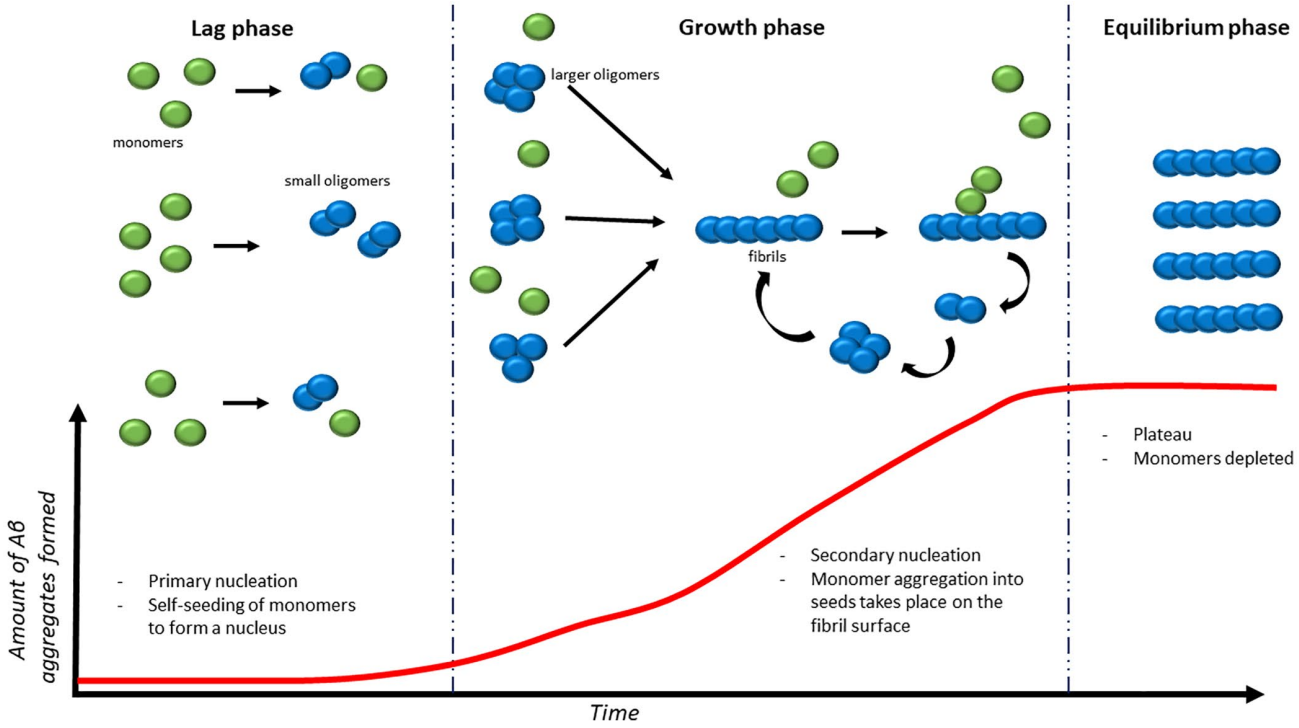

FIGURE 1 Nucleation, aggregation, and relationship of different beta-amyloid aggregation species. Aggregation of Aβ begins with the lag phase, during which monomers self-aggregate to produce nucleation seeds. This assembly is a slow, kinetically unfavourable process. A large number of initial nuclei is needed to produce small, then large oligomers, which eventually become elongated fibrils. During growth phase, the process enters secondary nucleation, where the aggregation of monomers into seeds is catalysed on the surface of the fibrils. This creates a positive feedback loop of aggregation, where small and large oligomers are constantly formed. These progress into fibrils, until a plateau is reached, when all monomers are depleted in an equilibrium phase

1.4 | The folding conundrum

Proteins and peptides that can form amyloids, including Aβ, exist as soluble monomers which are the smallest species that can aggregate to form insoluble fibrils, a process implicated in many diseases including Alzheimer's and Parkinson's disease (Cohen et al., 2013; Jan et al., 2011; Riek & Eisenberg, 2016).

Amyloid fibrils are the largest type of Aβ aggregates. Fibrils are formed of individual protofilament subunits of varying sizes and are characterised by their elongated and unbranched appearance composed of parallel β-sheets (Astbury et al., 1935; Colvin et al., 2016; Eisenberg & Jucker, 2012; Rambaran & Serpell, 2008). They bind to Congo red and Thioflavin T dyes due to their β-pleated sheet structure (Finder & Glockshuber, 2007; Sipe et al., 2016).

Amyloid fibrils are highly heterogenous, insoluble and non-crystalline (Colvin et al., 2016; Lührs et al., 2005; Rambaran & Serpell, 2008). Therefore, studying their structure via standard high-resolution techniques, such as X-ray diffraction or liquid state nuclear magnetic resonance (NMR) spectroscopy, can be difficult (Colvin et al., 2016; Lührs et al., 2005). Moreover, many solvents may change the aggregation pathway and kinetics of Aβ. This provides a further level of complexity when analysing in vitro and in vivo Aβ structures, as various preparation methodologies may not be interchangeable, and equally, may not produce the same aggregates.

It is thought that all fibrils originate from oligomers; however, Michaels et al. (2020) have suggested that oligomers may also dissociate into monomers without ever progressing into fibrils. It has also been suggested that only a minority of oligomers can proceed to fibril formation (Michaels et al., 2020).

The term 'oligomers' is broadly used and interpreted to include dimers through to heptamers, small oligomers, annular oligomers, globular oligomers, Amyloid β-Derived Diffusible Ligands (ADDLs) and protofibrils (Banerjee et al., 2017; Bruggink et al., 2012). These usually fall under a collective, umbrella term of 'oligomers', which describes different Aβ multimers. Unfortunately, the characterisation and nomenclature of the intermediate species, such as oligomers and protofibrils, are broad and often elusive (Rambaran & Serpell, 2008).

Aβ oligomers exist as heterogeneous species, consisting of populations of low molecular weight oligomers (less than 8 Aβ subunits) or high molecular weight oligomers (~42 kDa to 1 MDa in size) (Bruggink et al., 2012). Dimers can be described as aggregates with a diameter of ~3.5 nm (Finder & Glockshuber, 2007), whilst other reports state that oligomer diameters can range between 6 and 11 nm (Xue et al., 2019). Confusingly, large protofibrils have been characterised as ~5 nm in diameter, whilst fibrils are ~7–10 nm in diamater (Gremer et al., 2017; Yusko et al., 2012). Early publications characterised protofibrils as structures 6–8 nm in diameter and <200 nm in length (Walsh et al., 1997). Some publications classify protofibrils as high molecular weight Aβ oligomers (Ono & Tsuji, 2020), whilst others regard protofibrils as separate species entirely (Nichols et al., 2015). It is also possible that protofibrils are a component of an amyloid fibril.

Many studies refer to Aβ oligomers as 'on-pathway' and 'off-pathway', as well as 'non-fibrillar', 'pre-fibrillar' and 'fibrillar'. These terms may be used to describe the toxicity, structure, morphology and potentially aggregation kinetics of the oligomers. 'Fibrillar' oligomers have been described as 'off-pathway' and non-toxic oligomers, which can undergo indirect conversion into fibrils (via dissociation into monomers), whereas 'pre-fibrillar' oligomers have been described as 'on-pathway' neurotoxic intermediates of fibril aggregation (Verma et al., 2015). It has been shown that 'fibrillar' oligomers of $A\beta_{1\text{-}40}$ are fibrillar in structure, as they bind to the conformation-specific OC antibody, which recognises epitopes from fibrillary oligomers and amyloid fibrils, but does not recognise pre-fibrillar oligomers. Circular dichroism spectroscopy also reveals that fibrillar oligomers are ~20 nm, β-sheet rich particles. 'Fibrillar' oligomers can mimic the structure of mature fibrils and can also induce their formation. 'Non-fibrillar' oligomers of $A\beta_{1\text{-}40}$ are larger, globular oligomers which can vary in size between ~40 nm and ~60 nm. The 'non-fibrillar' oligomers can be detected by the conformation-specific A11 antibody. Circular dichroism spectroscopy revealed that non-fibrillar oligomers are mostly rich in random coils and α-helices (Vander Zanden et al., 2019).

Depending on the criteria and nomenclature used, the interpretation of the Aβ aggregate product may differ. Consequently, this may lead to contradicting results, hindering progression in this field. Standardisation of guidelines would help with the specific identification of peptide aggregate preparations and reproducibility of results. The field currently lacks consensus regarding the processes governing Aβ aggregation, plaque formation and toxicity of various Aβ species and isoforms. Moreover, the effects of in vitro and in vivo peptide environment on the Aβ aggregation status are poorly understood. This includes Aβ interactions with lipids, carbohydrates, small peptides, other proteins, cell membranes and metal ions as well as pH, ionic strength of the solution the aggregates are found in. Understanding how these processes occur is an important step in modelling the diversity of disease pathology and onset. It would also provide essential knowledge about the nature of heterogenic Aβ populations, which may impact toxicity and final structure of fibrils (Parodi-Rullán et al., 2020; Wulff et al., 2016). Interestingly, this heterogeneity of Aβ populations has been shown to differentially affect various cellular functions, depending on the Aβ species (Parodi-Rullán et al., 2020). It is entirely possible that components of such populations may play an important role in the degree of toxicity and AD pathology.

2 | Aβ TOXICITY AND METHODOLOGIES USED IN DISEASE MODELLING

2.1 | Overview

The amyloid hypothesis has undergone several modifications in relation to Aβ and the specific Aβ species implicated in AD pathogenesis (Benilova et al., 2012; Hardy & Higgins, 1992; Selkoe & Hardy, 2016). As opposed to amyloid plaques (and thus Aβ fibrils), the revised hypothesis implicates small, prefibrillar intermediate species of Aβ as the most neurotoxic species in AD pathology. The hypothesis is now

known as Aβ oligomer hypothesis. Research into toxicity of Aβ oligomers began in 1998 (Lambert et al., 1998). Since then, many studies have been published identifying Aβ oligomers as neurotoxic both in vitro and in vivo. In the brain, Aβ accumulates extracellularly, and it is assumed that binding of oligomers to plasma membranes is a driving force leading to the intracellular changes in AD (Yang et al., 2017). This may be an initiating step in AD onset and progression. Studies suggest that Aβ oligomerisation takes place at the plasma membranes (Zhang et al., 2012). However, the mechanism of Aβ toxicity, including oligomer toxicity in AD, is still unknown, and it is now becoming clearer that many factors may contribute to AD pathogenesis.

2.2 | Oligomers

Aβ oligomers may play multiple roles in AD pathology, including neuronal toxicity (Wicklund et al., 2010) and constriction of brain blood vessels (Nortley et al., 2019), excitotoxicity through elevated calcium levels (Arbel-Ornath et al., 2017; Resende et al., 2008), and promoting synaptic loss and inhibition of long-term potentiation (Shankar et al., 2007, 2008; Walsh et al., 2002). Moreover, Aβ oligomers can induce tau phosphorylation (Jin et al., 2011), as well as neuroinflammation by inducing a pro-inflammatory profile in glial cells (Maezawa et al., 2011; Michelucci et al., 2009; Sondag et al., 2009; White et al., 2005). Numerous studies have also demonstrated that plaque burden correlates poorly with cognitive impairment, and that toxic soluble oligomers correlate better with cognitive impairment (Haass & Selkoe, 2007; Mc Donald et al., 2010). Small soluble $A\beta_{1\text{-}42}$ oligomers can induce neurotoxicity both in vivo and in vitro, initiating the onset of AD. Hence, there is a current hypothesis that Aβ oligomers are more neurotoxic and more disease-relevant than Aβ fibrils.

The majority of Aβ species found in AD brain extracts contain high molecular weight aggregates, and these can dissociate into smaller oligomers and monomers. These small Aβ oligomers can inhibit synaptic dysfunction and stimulate microglial responses in vivo, further implicating low molecular weight oligomers as the more toxic species of Aβ (Yang et al., 2017). However, other studies found that brain-derived Aβ oligomers do not form smaller molecular weight aggregates, suggesting that dodecamers may be more relevant physiologically instead (Shea et al., 2019). Aβ is a self-aggregating protein, and oligomers are considered to be highly unstable, and therefore attributing toxicity to a specific species is difficult. It is especially true, as most Aβ preparation protocols can result in a heterogenous population of Aβ, which primarily varies in peptide size. It is therefore unclear as to which species are most pathologically relevant in AD (Verma et al., 2015).

2.3 | Aβ isoforms and toxicity

The clinicopathological phenotype of AD shows a large variability among patients, which includes varying age of onset, disease topography, genetic contributions and comorbidities (Lam et al., 2013). It has been suggested that AD phenotypes may be influenced by the patient's genetic background, as having specific mutation variants in AD-causative genes may affect both age of onset and the clinical presentation of AD (Ryan et al., 2016). The variety in AD clinical phenotypes can also be due to the co-morbidities present in most people with dementia (Matthews et al., 2009).

The heterogeneity in AD presentation may also be partly explained by Aβ polymorphisms and sequence isoforms. Many groups utilise different Aβ isoforms in their toxicity studies. The varying amino acid sequences of the peptides used in many studies ($A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42}$, N-terminally truncated Aβ) could result in changes to hydrophobicity, charge and polarity. This could affect the propensity for β-sheet formation and final peptide morphology (Klement et al., 2007; Rojas Quijano et al., 2006).

2.3.1 | $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$

$A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ are the most abundant and widely studied isoforms. The consensus in the field has been that $A\beta_{1\text{-}42}$ is the most toxic form of the peptide. However, this and the assumption that it is the β-sheet structure that is important for aggregation and hence toxicity are now known to be oversimplifications.

$A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ differ by two hydrophobic residues at the C-terminus, which are thought to confer the greater propensity to aggregate. Roychaudhuri et al. suggested that the propensity of Aβ to aggregate is linked to the structure conferred by the additional amino acids in the C-terminus. Residues 31–34 and 38–41 form a β-hairpin, reducing the flexibility within the C-terminus of the peptide, which then confers the propensity to aggregate (Roychaudhuri et al., 2013).

Both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ can induce cytotoxicity. $A\beta_{1\text{-}40}$ can induce a significant decrease in cell survival and increase in nuclear DNA damage in oligodendrocytes (Xu et al., 2001). $A\beta_{1\text{-}42}$ oligomers can induce neuronal death in the hippocampus in vivo, as well as reduce neuronal viability in vitro (Brouillette et al., 2012; Garwood et al., 2011). Although there is an increased loss of synapses in the vicinity of plaques, most synapse loss is observed near halos of oligomeric Aβ (Koffie et al., 2009) and there is a wealth of literature demonstrating that oligomeric $A\beta_{1\text{-}42}$ has a greater impact on neuronal loss and induces higher levels of apoptosis than fibrillar $A\beta_{1\text{-}42}$ (Doi et al., 2009; Resende et al., 2008).

Different oligomeric Aβ isoforms may cause varying effects on neuronal loss. Evidence suggests that $A\beta_{1\text{-}42}$ correlates better with synaptic loss than the shorter $A\beta_{1\text{-}40}$ (Bate & Williams, 2018). $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ isoforms can also differ in their ability to form aggregates. For example, $A\beta_{1\text{-}42}$ is more prone to aggregate (Iwatsubo et al., 1996), which could be a contributing factor to its toxicity in AD.

Post-translational modifications and truncations of Aβ can alter the aggregation process and confer toxicity. Furthermore, there is emerging evidence which challenges the notion that it is the β-sheet structure which drives aggregation and toxicity; Shea et al. (2019) describe how α-sheet Aβ oligomers form in the lag aggregation phase and that the emergence of these α-sheet structured oligomers, which precede the formation of β-sheet fibrils, correlates with Aβ-associated toxicity (Shea et al., 2019).

2.3.2 | N-terminally truncated Aβ

Although it is thought that $Aβ_{1-40}$ and $Aβ_{1-42}$ play a role in AD pathology, studies also implicate N-terminally truncated Aβ. In AD, amyloid plaques can consist entirely of full length $Aβ_{1-42}$ or of only N-terminally truncated $Aβ_{42}$, or a mixture of both (Saido et al., 1995). Previous work suggests that N-terminally truncated Aβ is one of the major components of amyloid plaques in AD (Naslund et al., 1994). The amount of N-terminally truncated Aβ significantly increases as AD progresses, and N-terminally truncated Aβ may also be more prone to forming toxic oligomers in AD (Meral & Urbanc, 2013).

N-terminally truncated Aβ is generated enzymatically by cleavage of the Aβ sequence at alternative sites by Beta-secretase 1 (beta-site APP cleaving enzyme 1, BACE1) generating $Aβ_{11-40}$ and $Aβ_{11-42}$, but there is further N-terminal heterogeneity which is thought to result from processing by alternative enzymes at different sites; the mechanism governing these are not well defined, nor are the sequences they generate. N-terminally truncated Aβ is thought have a particular propensity to form oligomers (Meral & Urbanc, 2013).

Amongst the N-terminally truncated forms of Aβ observed in AD brains, pyroglutamylated Aβ (Glu3) isoforms can be distinguished using mass spectrometry techniques (Mori et al., 1992). Formation of these Aβ isoforms involves a multi-step process, beginning with the removal of the first amino acids in the Aβ sequence, exposing the N-terminal glutamate. Glutamate is then post-translationally modified into N-terminal pyroglutamate by the enzyme glutaminyl cyclase (Jawhar et al., 2011; Nussbaum et al., 2012). MALDI-TOF mass spectrometry analysis of Aβ isolated from sporadic and familial AD cases indicates that, in addition to full length Aβ, there are also pyroglutamylated $Aβ_{3-42}$, pyroglutamylated $Aβ_{11-42}$, and N-terminally truncated $Aβ_{4-42}$ isoforms present in AD brains (Russo et al., 2000).

N-terminally truncated $Aβ_{4-42}$ causes dose-dependent neurotoxicity in vitro and induces memory deficits and neuronal loss in the hippocampus in vivo in transgenic mice expressing $Aβ_{4-42}$ (Bouter et al., 2013). Moreover, exposure to pyroglutamylated $Aβ_{3-42}$ causes significant neuronal loss at lower peptide concentrations than $Aβ_{1-42}$ (Nussbaum et al., 2012). It could be that post-translationally modified N-terminally truncated Aβ oligomers trigger hypertoxicity of full length $Aβ_{1-42}$ oligomers, in a prion-like manner (Nussbaum et al., 2012).

Even though they may play a key role in AD pathology, the structure and mechanism of toxicity of N-terminally truncated, as well as pyroglutamylated Aβ have not been studied as extensively as full-length Aβ species. The amount of pyroglutamylated Aβ is significantly increased in AD brains compared with control brains and correlates with Braak neurofibrillary tangle staging and severity of AD pathology. It has also been suggested that there may be a pathogenic link between pyroglutamylated Aβ and hyperphosphorylated tau in AD brains (Mandler et al., 2014).

Pyroglutamylated Aβ forms β-sheet structure more readily and has an increased predisposition to, and rate of, aggregation compared with full-length Aβ (Gunn et al., 2016; He & Barrow, 1999). This could be due to an increased hydrophobicity of the pyroglutamylated peptide (Goldblatt et al., 2017; Schlenzig et al., 2009). Pyroglutamylation of $Aβ_{3-x}$ involves the loss of two amino acid residues, which changes the properties of the peptide. Pyroglutamylated $Aβ_{3-40}$ rapidly aggregates into fibrils, which cannot be distinguished morphologically from $Aβ_{11-40}$ or full-length $Aβ_{1-40}$ fibrils. However, in contrast with $Aβ_{1-40}$ aggregation, the typical lag phase does not occur in pyroglutamylated $Aβ_{3-40}$ (Schlenzig et al., 2009) and is shorter for $Aβ_{11-40}$ aggregation, compared with full-length $Aβ_{1-40}$ (Barritt et al., 2017). Formation of $Aβ_{1-40}$ fibrils is accelerated in the presence of $Aβ_{11-40}$ (Barritt et al., 2017), indicating that not only can N-truncated Aβ, including pyroglutamylated Aβ, have a higher propensity to aggregate than full-length Aβ, but it will also modulate aggregation of the full-length Aβ isoforms.

A report by Gillman et al. (2014) suggests that not only is pyroglutamylated Aβ found in plaques, but it is associated with cytotoxicity, toxic oligomers and its levels correlate negatively with cognitive health (Gillman et al., 2014). One of the proposed mechanisms of Aβ toxicity is the disruption of cell membranes as a result of Aβ oligomers inserting into the membrane and forming pores (Bode et al., 2017; Serra-Batiste et al., 2016). Pyroglutamylated $Aβ_{3-42}$, as well as full length $Aβ_{1-42}$, can form pores in the lipid bilayer, but a longer time is required for the onset of pore activity for the pyroglutamylated $Aβ_{3-42}$ (Gillman et al., 2014). $Aβ_{1-42}$ has also been shown to produce the higher amount of ROS compared to pyroglutamylated Aβ. It may thus be that $Aβ_{1-42}$ is the most neurotoxic species of Aβ. However, compared with full-length $Aβ_{1-40}$ and pyroglutamylated $Aβ_{3-40}$, pyroglutamylated $Aβ_{3-42}$ produces higher amounts of ROS (Gunn et al., 2016). Moreover, pyroglutamylated $Aβ_{3-42}$ has been shown to induce significantly higher levels of membrane damage and lipid peroxidation in neuronal cultures than full-length $Aβ_{1-42}$ (Gunn et al., 2016). This could further suggest that $Aβ_{42}$ variants may be more neurotoxic than $Aβ_{40}$ variants.

The mechanism of Aβ toxicity in the brain is not yet fully understood and further work is needed to expand our current knowledge of neuronal loss in AD, as a response to various Aβ isoforms. It is clear that the identity of the most toxic Aβ species in AD is still unknown. It may be that multiple Aβ isoforms act in conjunction to elicit neurotoxicity and AD pathology. Therefore, studies should not only focus on the differences between different Aβ isoforms, but also on studying the effects of different Aβ forms collectively, as this would be the most likely mechanism of Aβ toxicity in the brain.

3 | Aβ PREPARATIONS

3.1 | Considerations

Research currently focuses on preparing controlled Aβ assemblies, which aids in studying their structure and function. Careful preparation of Aβ assemblies is crucial, as the peptide environment can influence Aβ structure influencing its function (Stine et al., 2011). Various protocols use solvents, such as hexafluoro-2-propanol (HFIP) and dimethyl sulfoxide (DMSO), as well as NaOH and NH_4OH at varying pH ranges. Such solvents could alter Aβ aggregation kinetics and

could produce non-physiological peptide structures (LeVatte et al., 2019).

Temperature, pH level, ionic strength, agitation, purity and concentration of Aβ used in the various protocols all contribute to the resulting peptide preparation (Stine et al., 2003, 2011). The ionic strength and pH of the solvent in which the Aβ is dissolved significantly affect the aggregation kinetics and hence the final aggregated structure (Lee et al., 2018; Xue et al., 2019). Temperature, hydrostatic pressure and solvents affect the physio-chemical environment of the peptide which impact its self-assembly (Klement et al., 2007). Furthermore, the amino acids present in the peptide sequence have varying hydrophobicity, charge and polarity, and these all affect whether the peptide folds into a β-sheet structure. Therefore, small amino acid differences in the various Aβ isoforms significantly impact the aggregation and the resulting morphology of the peptide (Klement et al., 2007; Rojas Quijano et al., 2006). Careful consideration needs to be given to the addition of salt ions to Aβ preparations since the addition of salts causes stronger self-aggregation resulting in shorter and less uniform fibrils. Moreover, the presence of membrane bilayers can catalyse self-aggregation of $A\beta_{1\text{-}42}$ oligomers, even at low concentrations of the peptide (Banerjee et al., 2020). Fibril morphology, Aβ aggregation and manipulation of production of toxic intermediates can also be dependent on the presence of endogenous or exogenous molecules in the Aβ environment (Soper et al., 2013). All these parameters could potentially impact in vitro experiments utilising cell culture, as well as studies of potential biotherapeutic agents for AD.

3.2 | Sources of Aβ

Furthermore, most investigations have utilised chemically synthesized Aβ peptides of varying lengths. The choice of Aβ source can be crucial when making observations in the context of Aβ biochemistry, properties, characterisation and disease relevance. Whilst we do not attempt to provide a comprehensive overview here, the prevailing finding is that oligomeric Aβ prepared from either synthetic or recombinant Aβ is significantly more toxic than fibrillar preparations, and that $A\beta_{1\text{-}42}$ confers greater toxicity than other isoforms without further post-translational modifications ($A\beta_{1\text{-}40}$, $A\beta_{25\text{-}35}$). Synthetic and recombinant Aβ may be used interchangeably in various studies. Synthetic Aβ peptides are readily available and widely used. However, combining the use of isotopically labelled synthetic Aβ with NMR-based techniques can be expensive, and therefore recombinant alternatives may be more appropriate (LeVatte et al., 2019).

As previously mentioned, Aβ aggregation is highly complex and studying Aβ aggregation has been challenging (Cohen et al., 2013). Amyloid aggregates can be synthesised in a laboratory setting by careful manipulation of protein concentration, solvent choice, pH, temperature and even choice of surface on which the peptide is allowed to aggregate (Hellstrand et al., 2010; Petkova et al., 2005; Stine et al., 2003; Walsh et al., 2009). Alterations in the peptide preparation can impact the aggregation, seeding and final morphology of the peptide product. Varying morphologies of Aβ can impact experimental outcomes, as it has been shown that different Aβ structural isoforms elicit varying toxicity on neurons in vitro (Petkova et al., 2005).

Another important issue which contributes to the variability in preparations is the presence of intrinsic impurities in synthetic Aβ preparations (Finder et al., 2010). Chemically sourced Aβ can have both contaminants and pre-aggregated forms of the peptide, and these can vary widely depending on the source of the peptide, the batch and storage and solubilisation conditions. Reproducible recombinant preparation methods need to be developed and optimised, and early issues relating to low yields, the production of unwanted Aβ variants and issues related to the production of the longer $A\beta_{1\text{–}42}$ need to be resolved. This adds a further layer of complexity as there are disparities in the aggregation propensity and toxicity of peptides derived synthetically to those derived using recombinant preparation methods, with recombinant $A\beta_{1\text{-}42}$ aggregating faster and being more neurotoxic than its synthetic counterpart (Finder et al., 2010). Recombinant preparations of Aβ can introduce truncations into the sample. Moreover, there can be batch-to-batch variations within the peptides.

3.3 | Cell-derived Aβ

To overcome some of the challenges of working with synthetic and recombinant forms of Aβ, researchers have increasingly sought alternative sources of Aβ for use in both in vitro and in vivo studies. One such alternative source comes from a modified Chinese hamster ovary cells stably expressing the Val717Phe human amyloid precursor protein line. These cells secrete Aβ in the form of monomers and 8–12.5 kDa Aβ-immunoreactive species,which immunochemical analysis and radio sequencing showed form dimers and trimers (Podlisny et al., 1995). The secreted Aβ is predominantly the $A\beta_{1\text{-}40}$ form and does not appear to aggregate beyond these smaller Aβ species. Dendritic spine loss, synaptic vesicle loss and perturbed hippocampal long-term potentiation (LTP) are observed in the picomolar to nanomolar range, a concentration thought to be similar to the levels present in AD brain. This differs from synthetic and recombinant Aβ preparations, which typically see these effects at concentrations in the micromolar range. More recently, it has been determined that these cells also secrete Aβ sequences that extend beyond Asp1, the conventional start point of the Aβ sequence. These are termed N-terminally extended Aβ and evidence suggests that these are more synaptotoxic than other species present (Welzel et al., 2014).

3.4 | Brain-derived Aβ

Recent studies have isolated Aβ from murine and human brain in order to better model the heterogeneity of the Aβ species present. Synthetic Aβ preparations do not cause seeding of Aβ in the same

manner observed in brain, suggesting that the misfolding of Aβ in vivo generates alternative conformations and/or that brain-specific cofactors are needed (Meyer-Luehmann et al., 2006). The addition of amyloid brain extracts to synthetic Aβ can accelerate the formation of Aβ fibrils (Paravastu et al., 2009). There is also a range of Aβ fibril polymorphisms in AD, and in vivo variations in Aβ fibril structures can influence variations of AD phenotype (Qiang et al., 2017). Moreover, it was shown that enriching $Aβ_{42}$ with small oligomers may increase the aggregation kinetics more than enrichment with larger oligomers (Xue et al., 2019), suggesting that low molecular weight oligomers may play an important role in nucleation polymerisation and fibril growth. This is because brain-derived Aβ can act as an initial 'seed' for the primary nucleation, which bypasses the need for a long lag phase, leading to more efficient aggregation. Interestingly, Aβ derived from different AD cases can propagate synthetic Aβ to aggregate into different Aβ 'strains' in vitro (Lu et al., 2013). These vary structurally and could also explain the clinical heterogeneity in terms of the clinical presentation of AD (Qiang et al., 2017).

Brain-derived Aβ fibrils differ from synthetic or recombinant Aβ fibrils structurally (Lu et al., 2013; Paravastu et al., 2008; Qiang et al., 2017). Brain-derived Aβ fibrils could be right-hand twisted, while the β-sheet of Aβ fibrils formed in vitro are left-hand twisted (Kollmer et al., 2019). It is therefore clear that there is not a single type of 'brain-derived Aβ'. in vivo studies have revealed that patterns of Aβ deposition vary in transgenic AD mouse models, and this may be dependent on the varying genetic background of the models (Langer et al., 2011). The advantage of using amyloid brain extracts when investigating the effects of Aβ in vitro and in vivo is that the Aβ has aggregated in pathologically relevant brain conditions. However, these extracts are not pure and can contain both fibrillar and non-fibrillar materials (Paravastu et al., 2009). Furthermore, brain-derived Aβ extracts can be composed of a mixture of Aβ isoforms (Kollmer et al., 2019). There may also be post-translational modifications present in the brain-derived Aβ extracts that will not be present in synthetic or recombinant Aβ preparations. These differences make it difficult to distinguish which species are pathologically relevant.

Extraction of Aβ from transgenic murine brain homogenates allows the sequential isolation of both soluble and insoluble Aβ (Casali & Landreth, 2016). Aβ isolated from brain is more resistant to enzymatic breakdown by proteinase K than synthetic $Aβ_{1\text{-}42}$, and this Aβ can also serve to seed further Aβ aggregation (Langer et al., 2011). Moreover, peptides enriched with Aβ from AD brains retain their physiological properties, as they can be internalised in vitro by cells (Pedrero-Prieto et al., 2019). The extraction of Aβ from human brain is becoming the gold standard approach, but this comes with considerable ethical implications. There also needs to be consideration of factors including post-mortem interval and brain pH, alongside the variability in the resulting preparations which cannot be accounted for through strict preparation protocols and those which arise from the inherent genetic variability in humans.

4 | TOOLS FOR ANALYSIS

Several approaches can be used in the analysis of various Aβ species, all of which have their advantages and disadvantages. Therefore, a combination of approaches is needed to accurately analyse the species of Aβ within a defined preparation. Most commonly, microscopy techniques are used to reveal the morphology of the peptide in question (Rambaran & Serpell, 2008). Amyloid fibrils can bind Congo red dye and can show cross β-sheet X-ray diffraction patterns (Sipe et al., 2016). Other biophysical tools are available to study fibril structures, such as NMR and circular dichroism (CD) (Rambaran & Serpell, 2008). Mass spectrometry analyses can also be utilised. These are useful when determining the identity of Aβ isoforms and aggregates present in a heterogeneous preparation, such as those derived from AD brain tissue (Kollmer et al., 2019).

4.1 | Microscopy

Multiple approaches can be taken to study Aβ morphology and structural conformation. High-resolution microscopy techniques, such as transmission electron microscopy (TEM) and atomic force microscopy (AFM), are used to visualise and analyse Aβ aggregation states (Bruggink et al., 2012). Such analyses are often supplemented by other techniques to separate and quantify the different species, revealing the most information about the structure, molecular weight and concentration of Aβ aggregates.

Microscopy can reveal morphological differences between Aβ preparations. Aβ fibrils prepared in vitro are reported to follow a left-hand twist structure, as shown by both AFM and EM analyses (Kollmer et al., 2019; Sachse et al., 2006; Schmidt et al., 2015), while ex vivo formed Aβ fibrils may have a right-hand twist structure (Kollmer et al., 2019). Twisting of amyloid fibrils can depend on experimental conditions, and twisting variability may be present in a single fibril structure (Periole et al., 2018). Such polymorphisms may be the cause of variations in AD clinical and pathological phenotypes (Qiang et al., 2017).

TEM can be used to analyse Aβ fibrils derived from brains of AD patients. This has shown that patients can develop structurally different fibrils, suggesting the presence of Aβ fibril polymorphisms (Lu et al., 2013). Cryo-EM is a variant of TEM, which uses cryogenic temperatures that enable better preservation of protein structures (Bruggink et al., 2012). Cryo-EM studies have revealed that the structure of $Aβ_{1\text{-}42}$ fibrils prepared in vitro is long, unbranched, and composed of two intertwined protofilaments arranged into parallel cross-β strands (Gremer et al., 2017). Furthermore, cryo-EM can highlight the polymorphism of brain-derived Aβ fibrils. Whilst being polymorphic, brain-derived Aβ can contain structured protofilaments that are similarly structured. The structures of brain-derived Aβ fibrils and fibrils formed in vitro varies greatly (Kollmer et al., 2019). This could potentially lead to differing cell responses when varying structures of Aβ are

used in experimental design. Cryo-EM has recently been coupled with the use of nanoparticles, which attach to and label amyloid fibrils. This technique enabled quantitative image analysis of such fibrils, improved contrast, and provides a new and efficient tool for rapid detection and characterisation of Aβ polymorphisms (Cendrowska et al., 2020). Cryo-EM technique can be supplemented by NMR spectroscopy, to obtain a 3D model of $A\beta_{1\text{-}42}$ fibril conformation (Gremer et al., 2017). Supplementing NMR analyses with EM can also provide essential information about the difference in amino acid residues of *ex vivo* fibrils obtained from different AD brains. These fibrils can vary in residue interactions and backbone, but not in the overall symmetry of the peptide (Lu et al., 2013).

AFM is a high-resolution technique, enabling the reconstruction of 3D polymer morphology (Ruggeri et al., 2019). AFM is capable of measuring the growth of Aβ fibrils (Xu et al., 2019), as well as studying the structure of Aβ oligomers, particularly with regards to N- and C-termini (Yoon et al., 2019). Time-lapse imaging using AFM can reveal the morphology and aggregation state of oligomeric preparations under various conditions (Banerjee et al., 2020). AFM requires a smaller sample concentration and volume and can produce more detailed analyses than EM (Bruggink et al., 2012). In comparison to EM, samples analysed through AFM do not have to be stained or frozen, and therefore can be studied in more physiological conditions (Dasgupta et al., 2020). As oligomers are smaller than fibrils, there is a need for high-resolution techniques such as AFM. However, AFM is often carried out after drying the sample, so that the sample remains static. Therefore, AFM may not be very representative of peptides in solution, which could be argued to be more physiological.

High speed AFM can be used to provide structural and kinetic information about various conformations of Aβ in vitro. For example, single-molecule interactions between different Aβ species can be detected, which enables the quantification of oligomerisation kinetics (Feng et al., 2019). High-speed AFM analysis of Aβ oligomers has shown that low molecular weight oligomers are largely globular, with pentamers and heptamers being the most dynamic structures (Banerjee et al., 2017). High-speed AFM analysis has also revealed that Aβ monomers and low molecular weight oligomers can aggregate dynamically via three distinct pathways, to produce high molecular weight oligomers (which do not progress into fibrils), 'spiral' fibrils and 'straight' fibrils, which can all result in different peptide dynamics and polymorphisms. These pathways can be influenced by the peptide environment, such as the composition of aggregation buffers (Watanabe-Nakayama et al., 2016). Moreover, AFM has revealed more information about the importance of Aβ N- and C-termini. The N-terminus could be an important player in mediating Aβ aggregation into more toxic species by modulating oligomerisation, whilst the C-terminus may affect the stability of the peptide, as well as how monomers interact with other Aβ aggregates (Foroutanpay et al., 2018; Lv et al., 2013).

4.2 | Circular dichroism

CD spectroscopy is a widely used and validated technique used for the analysis of Aβ tertiary structure. The CD spectrum in the far UV region can identify different conformations of peptides, such as random coils, α-helices and β-sheets (Bruggink et al., 2012). During aggregation, Aβ transitions from a random coil or α-helix structure to a β-sheet conformation. This is indicated in CD by a decrease in signal at 218 nm (Bruggink et al., 2012; Sachse et al., 2006). Plotting the signal changes over time can reveal a sigmoid aggregation curve, corresponding to the different Aβ aggregation phases (Figure 1) (Bruggink et al., 2012). This is consistent with results obtained through the Thioflavin T (ThT) assay, which can track Aβ aggregation into β-sheet rich structures (Bruggink et al., 2012; Nilsson, 2004; Sachse et al., 2006).

4.3 | Nuclear magnetic resonance spectroscopy

Because Aβ aggregates are non-crystalline and insoluble, it can be difficult to study their structure, as many traditional methods such as X-ray crystallography and direct liquid-state NMR are not appropriate (Tycko, 2016). Solid-state NMR (ssNMR) is a quantitative technique able to look at both the structure and dynamics of compounds. NMR spectroscopy uses radio waves to irradiate atomic nuclei, and the corresponding resonance frequency is recorded. The frequency of irradiation depends on the magnetic strength of an isotope. The NMR spectrum provides information about the chemical shift, which can determine the content and molecular structure of the sample. ssNMR has been shown to be a useful tool in the analysis and modelling of Aβ fibril β-sheet conformations, also revealing the dynamics of fibrils particularly at the N- and C-termini (Petkova et al., 2002; Scheidt et al., 2011, 2012). ssNMR can also be used in vitro to study different growth conditions and subsequent varying Aβ fibril morphologies (Paravastu et al., 2008, 2009). ssNMR is an appropriate technique to study fibril structure at atomic resolutions and can be used in conjunction with microscopy techniques. However, to generate high-resolution ssNMR spectra, a monomorphic preparation of amyloid fibrils is required. Therefore, to obtain such results, careful preparations of Aβ aggregates are needed to derive highly pure end products (Colvin et al., 2016).

Pressure-jump NMR is an NMR-based technique that can be used to study the kinetics of Aβ oligomerisation and to observe Aβ aggregation (Barnes et al., 2019). It is a very informative technique used for studying protein assembly, especially with Aβ where oligomers can develop in seconds (Barnes et al., 2019). Pressure-jump NMR has revealed that formation of protofibrils is a slow kinetic process, which can be manipulated by pressure changes. Protofibrils can dissociate into monomers under high pressure, while under low pressure these monomers can re-associate into protofibrils (Kamatari et al., 2005). Similar to protofibrils, $A\beta_{40}$ oligomers dissociate at higher pressure and aggregate at lower pressure. These changes can be tracked by recording the NMR spectra, which have shown that $A\beta_{40}$ oligomers

do not contain a regular amyloid fibril structure, but rather contain antiparallel β-strand arrangement. NMR techniques can therefore be very informative, as they facilitate studying amyloid aggregation at residue-specific resolution (Barnes et al., 2019).

4.4 | Dye-based assays

ThT assays are commonly used to detect Aβ fibril formation in vitro over time (Nilsson, 2004). ThT assays detect changes in fluorescence intensity when ThT binds to amyloid fibrils, revealing the presence of β-sheets (Lührs et al., 2005; Nilsson, 2004). Fluorescence can be detected when the fibril structure is rich in β-sheets (Hudson et al., 2009). Sigmoidal kinetic curves are observed corresponding to the nucleation polymerisation pathway of Aβ (Figure 1) (Shea et al., 2019). Congo Red is another dye, which has been used as a marker for amyloid fibrils *ex vivo*. The presence of amyloid fibrils results in a light absorption at ~490 to ~540 nm (Girych et al., 2016).

Dye-based kinetic assays such as ThT are simple and can be carried out in a microplate, allowing a high-throughput analysis of Aβ fibril formation (Girych et al., 2016; Sebastiao et al., 2017). However, there are potential drawbacks when using these methods, especially when monitoring the impact of potential drugs on fibril formation. Exogenous compounds, such as curcumin and resveratrol, interfere with ThT fluorescence and introduce biases in the analysis (Hudson et al., 2009). Therefore, ThT assays should always be supplemented with other analysis methods such as EM and Congo Red binding assays. Combining both Congo Red and ThT assays can have advantages over single-dye assays, as this can minimise false positive and false negative results (Girych et al., 2016). Moreover, there is currently lack of consensus in the literature regarding appropriate ThT concentrations used to detect amyloid fibrils. ThT auto-fluoresces at concentrations above 5 μM; therefore, when higher concentrations are used, it is necessary to incorporate appropriate background corrections in the experimental design. It is suggested that the optimal ThT concentration for Aβ fibril detection should always be below 50 μM, as concentrations of 50 μM and higher affect peptide aggregation rates (Xue et al., 2017). These assays are relatively inexpensive, easy and fast to carry out and therefore should be incorporated as a standard test for all fibril preparations.

A combination of circular dichroism and ThT techniques has been implemented to study the progression of Aβ fibrils from a random coil conformation to β-sheet structure. Circular dichroism and ThT can be used to monitor the structural changes of oligomers, revealing that oligomers present in the lag phase (Figure 1) do not contain a conventional secondary structure; however, higher molecular weight oligomers can adopt a β-sheet conformation (Shea et al., 2019). Fast and dynamic spectroscopy-based techniques can reveal essential information about the peptide structures; however, they lack the ability to directly visualise them (Feng et al., 2019). Therefore, it is essential to combine techniques together to obtain as much information about the aggregate species as possible. For example, a combination of ThT and EM techniques has been used to determine the effect of oligomer heterogeneity on fibril formation. This has revealed that the heterogeneity of oligomers may be a key determining factor leading to fibril polymorphisms (Xue et al., 2019).

4.5 | Size exclusion chromatography

Size exclusion chromatography (SEC) enables separation of particles by their size, and in some cases by molecular weight. A combination of size exclusion chromatography SEC, EM and light scattering have been in use for decades (Walsh et al., 1997). These have, for example, enabled the detection and characterisation of Aβ protofibrils (Walsh et al., 1997), as well as enabling the analysis of structural differences between Aβ protofibrils and oligomers (Nichols et al., 2015). Furthermore, SEC can be used to separate high molecular weight oligomers from low molecular weight oligomers, and the eluted fractions used for further analysis. This is particularly useful when using Aβ obtained directly from human brain homogenates, where TBS-soluble Aβ is isolated in both oligomeric and fibrillar forms (Yang et al., 2017). SEC has been used to analyse molecular weight distributions of oligomers (Shea et al., 2019) and to isolate monomeric Aβ fractions. In the past, the eluted materials were collected and subjected to further analysis, for example via Sodium Dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Bitan et al., 2003; Fukumoto et al., 2010). However, there are many drawbacks to this technique, which may interfere with the protein aggregation (Pujol-Pina et al., 2015). Dynamic light scattering can follow SEC analysis, and this can reveal the size of particles isolated. This can be used to supplement data obtained through other methods of analysis, such as EM and SDS-PAGE (Bitan et al., 2003). However, adsorption of aggregating species to SEC columns can make the application of this technology limited. SEC has been used to separate high molecular weight from low molecular weight oligomeric species. This is particularly useful when analysing Aβ obtained directly from human brain homogenates. Nevertheless, the impact of the column matrix on different Aβ assemblies is difficult to evaluate.

4.6 | SDS-PAGE

To study heterogenous Aβ aggregates via SDS-PAGE, peptides have to be photochemically cross-linked in order to achieve their appropriate separation via size (Banerjee et al., 2017; Rahimi et al., 2009). The method, photo-induced cross-linking of unmodified proteins (PICUP), works by stabilising Aβ aggregate populations by covalent cross-linking. PICUP can be used to separate generated Aβ oligomer preparations by size using both SEC and SDS-PAGE, which can then be used in appropriate experimental designs (Banerjee et al., 2017; Rahimi et al., 2009). This enables visualisation and quantitative analysis of the peptides. PICUP and SDS-PAGE are routinely used in the literature, often as the only method of analysis of Aβ preparations. However, SDS can affect the oligomerisation state of the peptides, producing oligomer artefacts and leading to unreliable interpretation of results (Pujol-Pina et al., 2015). It is therefore imperative to

supplement SDS-PAGE data with other, more reliable techniques, which provide insight into the size distribution as well as the morphology of Aβ preparations, at a higher resolution. SDS-PAGE and western blotting can however be performed to confirm the presence of Aβ in a preparation (Kollmer et al., 2019), and this is particularly important when analysing preparations obtained from AD brain tissue.

4.7 | Mass spectrometry

Identification and characterisation of individual Aβ species can be a major challenge, especially when oligomers of varying sizes, as well as Aβ protofibrils and fibrils can co-populate a single solution (Scarff et al., 2016). Several mass spectrometry (MS)-based platforms have been created and utilised to study such heterogeneous peptide mixtures. MS approaches are high-throughput, fast and robust. MS can be used at femtomolar concentrations, separating and identifying peptides based on their mass-to-charge ratios (m/z) (Scarff et al., 2016). Proteins studied via native MS methods can retain many aspects of their native structure because native MS uses gentle conditions in a gas phase. This preserves noncovalent interactions of peptides, allowing for analysis of complex stoichiometries, protein modifications, overall protein shape, relationships between binding agents and ligands, and protein-protein interactions (Lermyte et al., 2019; Österlund et al., 2019; Young et al., 2015). MS also allows for characterisation of individual amyloid species formed during the aggregation process (Smith et al., 2006). Moreover, due to its high resolution, MS could also be applied to study Aβ aggregation in physiologically relevant environments, such as in the presence of metal ions (Zhang et al., 2013). When coupled with other methods, such as ion mobility (IM), electrospray ionisation (ESI) and matrix-assisted laser-desorption ionisation (MALDI), these methods can be a powerful tool for protein analysis, especially considering proteins of high complexity and disease relevance, such as Aβ (Verbeck et al., 2002). Combining MS-based techniques together could allow for a well-rounded analysis of the Aβ peptide.

Ion mobility mass spectrometry (IM-MS) can be used to provide information about protein conformations as well as protein oligomeric states (Österlund et al., 2019; Verbeck et al., 2002). Moreover, any changes to the oligomeric states, conformation and aggregate formation can be confidently monitored over time (Scarff et al., 2016). IM-MS requires a comparatively low concentration of sample to record the size and shape of peptides (Scarff et al., 2016; Soper et al., 2013). Furthermore, IM-MS has been suggested as a valuable tool for investigating and detecting dimer-related complexes, which may not be possible with other MS-based approaches (Soper et al., 2013). IM-MS was able to provide valuable information about Aβ conformations, showing that the structures of $A\beta_{40}$ and $A\beta_{42}$ oligomers can vary greatly. This can also have an impact on the amount of Aβ oligomers formed, and their varying morphologies produced further in the aggregation process (Österlund et al., 2019). Different $A\beta_{42}$ isoforms can also show many morphological differences. IM-MS experiments have revealed that every $A\beta_{42}$ oligomer has two arrangements, which can stem from different tertiary or quaternary structures of oligomers, leading to Aβ aggregates adopting several conformations. Coupling IM-MS experiments with NMR and cryo-EM measurements showed that Aβ oligomers can adopt at least two growth models (globular or linear growth), depending on their structural arrangements. This can further influence the aggregation pathway, leading to formation of fibrils or amorphous aggregates (Lieblein et al., 2020).

IM-MS can also be used to study potential therapeutic targets for AD. IM-MS studies revealed leucine enkephalin as an agent able to bind to and form a complex with Aβ. Combining IM-MS with TEM further revealed that such binding can inhibit Aβ aggregation into fibrils, which is the main component of amyloid plaques in AD brains (Soper-Hopper et al., 2017). IM-MS has also been used as a tool for screening for potential neuropeptides able to bind with and modulate aggregation and stoichiometries of Aβ forms (Soper et al., 2013). Moreover, IM-MS was used to assess the effects of C-terminal fragments (CTF) on Aβ aggregation, revealing subtle differences in Aβ oligomerisation pathways, which could be linked to modulating Aβ-induced toxicity (Gessel et al., 2012). Utilising MS-based techniques in tandem with other classically used techniques such as microscopy could therefore be a useful approach not only to study the molecular and biophysical properties of various Aβ aggregates, but also for studying and monitoring the Aβ aggregation process itself. Such tools are important for the development of potential AD therapies, particularly in the context of small peptide-based amyloid inhibitors.

Matrix-assisted laser-desorption ionisation (MALDI) MS is compatible, and often used in conjunction with other MS techniques, which can provide a more comprehensive peptide analysis. The data produced via MALDI-MS are relatively easy to interpret when compared with other MS-based methods. Time of flight (TOF) analysers are often used in conjunction to determine the mass/charge (m/z) ratio (Singhal et al., 2015). As with other MS-based techniques, MALDI-MS is useful for detection of low amounts of proteins and peptides in samples and can be used as a more reliable alternative to antibody-based techniques (Kakuda et al., 2017; Kaneko et al., 2014; Pekov et al., 2019). Ikegawa et al. (2019) developed a protocol coupling MALDI with ionisation-based mass spectrometry (IMS) and used this technique to obtain comprehensive protein information on human autopsy brain tissue (Ikegawa et al., 2019). This allowed a detailed visualisation of Aβ deposits in AD brains, revealing that, although predominant, $A\beta_{1\text{-}42}$ is not the only Aβ variant present in senile plaques. N-terminally and C-terminally truncated Aβ, as well as modified Aβ are also present in pathological deposits. Thus MALDI-MS is a valuable tool aiding standardised techniques such as immunohistochemistry. A major advantage MALDI-MS is that it does not require specific antibodies to differentiate Aβ species in the brain, allowing a more comprehensive analysis of the deposits (Ikegawa et al., 2019), and it can aid in localising and distinguishing between different truncated forms of $A\beta_{42}$ and $A\beta_{40}$ in brain amyloid deposits in AD (Kakuda et al., 2017). MALDI-TOF MS was able to determine Aβ isoform pattern in plaques located in various AD brain

regions, showing that pyroglutamated Aβ, N-terminally truncated Aβ, and $Aβ_{1-42}$ correspond well to plaques and may exhibit higher aggregation propensity, higher neurotoxicity in AD than other Aβ isoforms (Portelius et al., 2010). Furthermore, coupling MALDI technique with TOF/TOF allows for a detailed, rapid and sensitive analysis of post-translational modifications of Aβ, which may not be detected via standard methods that utilise widely used antibodies, such as 6e10 (Pekov et al., 2019). MALDI- TOF/TOF analysis of Aβ peptides originating from iPSC-derived neurons, coupled with immunoprecipitation, was able to reliably quantify Aβ levels in cells, and confirm that fAD mutations, and their subsequent effects on APP, can influence Aβ42:40 ratios (Arber et al., 2020). MALDI-MS can be expensive and often relies on pre-preparation steps. Moreover, it cannot distinguish molecules with overlapping m/z ratios, so may not be appropriate for analysing heterogenous Aβ populations formed during the aggregation processes (Pryor et al., 2012).

Electrospray ionisation (ESI)-MS is another label-free MS-based tool of analysis for Aβ. ESI-MS can be coupled with IMS to account for the fact that oligomers of different sizes can exhibit the same m/z peaks. ESI-IMS-MS enables study of oligomers based on their shape, stoichiometry, stability, assembly and binding affinity (Scarff et al., 2016). ESI has a wide scope of applications in research, is fast, and uses small amounts of sample (Skribanek et al., 2001). ESI can also be utilised in investigating potential therapeutics for AD by revealing interactions of Aβ peptide with small molecules acting as aggregation inhibitors (Skribanek et al., 2001). A variant of ESI-MS, called cold spray ionisation mass spectrometry (CSI-MS) is an alternative method. It proved to be a milder and less destructive method, advantageous when studying non-covalent drug-protein stoichiometries, and thus could be a more appropriate approach for many aspects of Aβ therapeutics research (Jiang et al., 2019; Zhou et al., 2020).

When ESI is coupled with MALDI, they form a simpler MS-based technique capable of differentiating variants of Aβ oligomers obtained after oligomerisation incubation. However, it is worth noting that ESI-MALDI is not a fully quantitative analysis tool and rather produces a more qualitative result. Regardless of that, ESI-MALDI could be a powerful method allowing researchers to confirm the Aβ species and aggregates produced during their experimental design (Wang et al., 2018). Fiori et al. (2013) utilised both ESI-ion trap-MS, as well as MALDI-TOF-MS to gain a comprehensive insight into the aggregation of Aβ25-35 peptide fragment. Combining both MS-based methods, a robust and reproducible approach could be developed, which allowed the investigation of peptide self-assembly and inhibition (Fiori et al., 2013).

MS-based methods can also be applied as an alternative method to immunoassays when studying and detecting Aβ in the cerebrospinal fluid (CSF). Widely used antibody-based immunoassay approaches, such as ELISA, depend on the use of specific antibodies leading to variability in results (Pottiez et al., 2017). Immunoassays for Aβ detection in human samples are flawed, as they can vary in epitopes as well as antibodies. Immunoassays can also lack standardised reference material, and standardised protocols and methodologies. The variability of results in such methods was shown to be too high to allow a specific and universal biomarker, and any associated cut-off values, to be identified. (Mattsson et al., 2011, 2013). To solve this issue, non-immunological, MS-based methods can be used (Korecka M et al., 2014; Mattsson et al., 2011; Pottiez et al., 2017). Such methods can be used not only for evaluating Aβ levels in human samples, but also for evaluating another protein of importance in AD, tau, in tandem in a single experiment (Pottiez et al., 2017). Moreover, plasma $Aβ_{40/42}$, analysed using liquid chromatography mass spectrometry, has been shown to accurately diagnose brain amyloidosis, and quantify both $Aβ_{1-40}$ and $Aβ_{1-42}$ in the CSF. MS-based techniques can also be used to screen individuals at risk for AD and to identify AD biomarkers (Lim et al., 2020; Schindler et al., 2019; Seino et al., 2021; Weber et al., 2019). This could ignite a potential for MS-based techniques to be used as a standardised diagnostic tool for AD, as well as a screening tool for clinical trials.

5 | CONCLUDING REMARKS

The biochemical complexity of Aβ makes experimental design difficult. Although there have been significant advances in our understanding of Aβ aggregation kinetics and chemistry, the field still suffers from a lack of standardisation in methods of characterisation, nomenclature, analytical approaches and in vitro models of Aβ.

There is a variety of biochemical and biophysical methods available to allow investigation of the structure and properties of Aβ. Novel technologies are currently being developed, and many techniques are used in combination to improve comparability and reproducibility of studies. Further development, standardisation and improvement of existing protocols are absolutely necessary to understand the biology of Aβ, its role in disease, and potential therapeutic agents tackling this complex field.

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CONFLICTS OF INTEREST

No conflicts of interest to disclose.

AUTHORS' CONTRIBUTIONS

Ferraiuolo L., Simpson JE., Staniforth RA. - provided guidance and feedback over the manuscript; Garwood CJ. - provided guidance and feedback, as well as re-written sections, and provided references and planned content for the manuscript. Wharton SB. - provided guidance, feedback, references and comments for the manuscript, as well as provided edits to sections of the manuscript and planned the contents of the manuscript. Matuszyk MM.- Planned content for

the manuscript, researched references, made figures and has written the review.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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