

Review

Amyloid- β Oligomers: Multiple Moving Targets

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Abstract: Alzheimer's Disease (AD) is a neurodegenerative disorder that is characterized clinically by progressive cognitive decline and pathologically by the β -sheet rich fibril plaque deposition of the amyloid- β (A β) peptide in the brain. While plaques are a hallmark of AD, plaque burden is not correlated with cognitive impairment. Instead, A β oligomers formed during the aggregation process represent the main agents of neurotoxicity, which occurs 10–20 years before patients begin to show symptoms. These oligomers are dynamic in nature and represented by a heterogeneous distribution of aggregates ranging from low- to high-molecular weight, some of which are toxic while others are not. A major difficulty in determining the pathological mechanism(s) of A β , developing reliable diagnostic markers for early-stage detection, as well as effective therapeutics for AD are the differentiation and characterization of oligomers formed throughout disease propagation based on their molecular features, effects on biological function, and relevance to disease propagation and pathology. Thus, it is critical to methodically identify the mechanisms of A β aggregation and toxicity, as well as describe the roles of different oligomers and aggregates in disease progression and molecular pathology. Here, we describe a variety of biophysical techniques used to isolate and characterize a range of A β oligomer populations, as well as discuss proposed mechanisms of toxicity and therapeutic interventions aimed at specific assemblies formed during the aggregation process. The approaches being used to map the misfolding and aggregation of A β are like what was done during the fundamental early studies, mapping protein folding pathways using combinations of biophysical techniques in concert with protein engineering. Such information is critical to the design and molecular engineering of future diagnostics and therapeutics for AD.

Keywords: Alzheimer's Disease; amyloid- β ; oligomers; α -sheet



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1. Introduction

Alzheimer's Disease (AD) is a fatal neurodegenerative disorder clinically characterized by the progressive deterioration of memory and cognitive functions. It is the sixth leading cause of death in the US and the leading cause of dementia worldwide, affecting more than 50 million people [1–3]. The primary pathological indicators of AD are extracellular amyloid- β (A β) plaques and intraneuronal neurofibrillary tangles of the tau protein [2]. The cascade of plaque deposition and tangle formation follows a pattern: starting in the entorhinal/perirhinal cortex, spreading through limbic structures and the hippocampus, and eventually reaching the frontal, temporal, and parietal cortex [4]. The process begins with the misfolding of the A β peptide, which is clipped from the amyloid precursor protein (APP) by α -, β -, and γ -secretase enzymes. In its monomeric form, A β is associated with a variety of biological functions [5–10], but it can misfold into an aggregation competent state, leading to a heterogeneous distribution of low- to high-molecular-weight oligomers that eventually form the characteristic amyloid plaques [11]. Importantly, disease progression is not correlated with amyloid plaque burden nor tau tangle formation, but rather with the presence of low molecular weight (LMW) soluble oligomers that act as the primary toxic agents [12–32]. In fact, in the absence of fibrils, these LMW soluble oligomers induce toxicity and neuronal death, as demonstrated in mouse models of AD [21,26] and familial

cases of AD that do not produce plaques [27]. Additionally, tau-mediated neuronal injury is a downstream component of AD progression preceded by A β accumulation and synaptic dysfunction, and A β oligomerization is the first known biochemical change that occurs (Figure 1) [28–60]. Over the past decades, researchers have pursued various avenues to gain a molecular level understanding of A β misfolding and its relation to toxicity as the primary agent in AD pathogenesis [59–66].

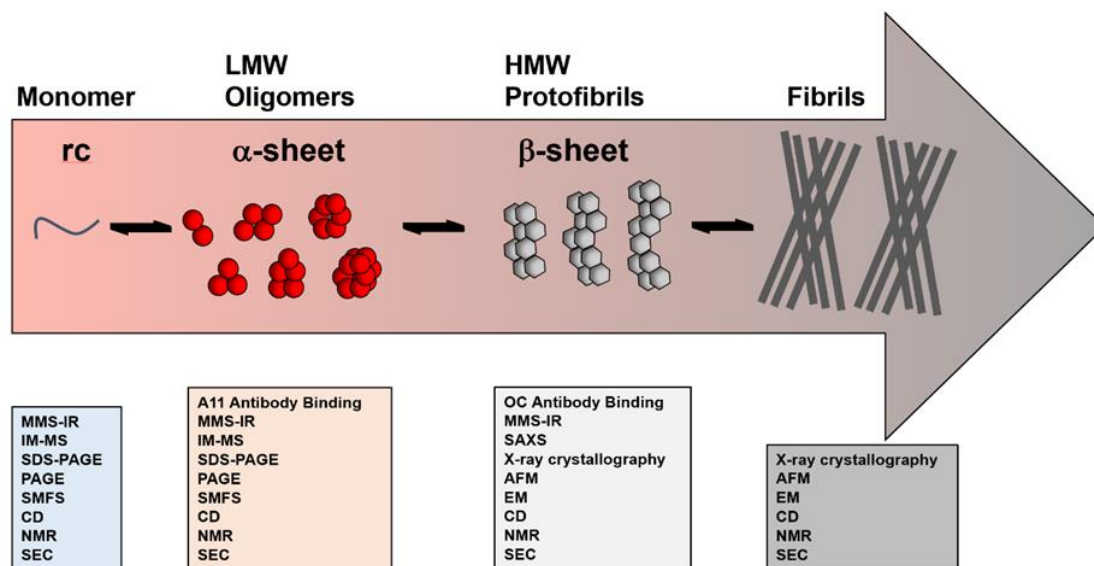


Figure 1. Pathology of disease progression and characterization techniques. Low molecular weight aggregates are soluble and contain primarily α -sheet structure that have been characterized by size and structure by the associated techniques. High molecular weight aggregates are soluble but contain primarily β -sheet structure that can be characterized by the associated techniques.

Many labs have shown that LMW A β oligomers are more cytotoxic than protofibrillar and fibrillar structures and that they inhibit critical neuronal activities, including long-term potentiation (LTP), a classic model for synaptic plasticity and memory loss *in vivo* and in cell cultures [21,63,67,68]. Isolated soluble oligomer aggregates from *in vitro* and/or *in vivo* sources range in size from dimers to 24-mers, to even higher-ordered assemblies moving through the aggregation process [69–72]. However, some techniques may bias the oligomer profile, thereby making it difficult to correlate what is observed in the lab with what is present in the brain [73]. In this review we present studies on the oligomerization process—as characterized by a range of methods—to delve into the role that A β oligomers play in toxicity, the techniques that help to characterize different oligomer assemblies, and how this information can be used to better interpret the broad spectrum of A β -related data and propel AD research toward a potential treatment.

2. Aggregation and Characterization

Monomeric A β (4.5 kDa) is produced by the sequential proteolytic cleavage of APP (120 kDa) by β -secretase and γ -secretase in endosomes and at the plasma membrane [74]. α -Secretase also presents a pathway for formation of shorter A β fragments that are thought to be non-amyloidogenic [75]. Despite their common origin, the variants each possess different solubilities, stabilities, and biological and toxic properties. Clipping at the C-terminus by the γ -secretase results in variants ranging from A β 43, A β 42, A β 40, A β 38, and A β 37 (where the number indicates the length of the fragment, ex. A β 43 is residues 1–43), which are detected in cell culture and body fluids [3,12,15–17]. Further heterogeneity is exacted by diverse enzymatic processes by aminopeptidases, glutaminylicyclase, isomerases, and phosphorylation reactions, which all contribute to the sprawling list of 20 A β peptides that contribute distinctly to the intrinsic A β functions in the normal brain as well as aggregation

and toxicity in the AD brain [75,76]. Of this list, A β 40 is the most constitutively produced fragment in both healthy and AD patients, while the other A β peptides are continuously produced, but at lower levels.

Despite A β 40 being the most abundant form, it is not the most pathologically relevant. Rather, A β 42—with two additional hydrophobic residues Ile41 and Ala42 at the C-terminus—comprises the majority of plaque deposits, can seed fibril formation, and more robustly induces toxicity than its counterparts [77–79]. Additionally, there are distinct kinetic characteristics for the aggregation of A β 40 and A β 42, as well as different toxicity profiles and altered behavior when the two are co-incubated [80,81]. A common biomarker to confirm clinical AD diagnoses includes the ratio of the concentrations of A β 42/A β 40, which in the early stages of the disease is relatively high but then decreases as the disease progresses and more A β 42 is deposited into the plaques. In addition to the complex interplay between A β variants, oligomer formation can occur through both primary and secondary nucleation, involving a pool of A β monomers assembling, or monomers that interact with A β aggregates to produce higher-order aggregates, respectively [82]. Worthy of Heraclitus's Law of Change, this results in a wildly heterogeneous and dynamic distribution of oligomer species in a state of constant change.

Due to the dynamic nature of these A β aggregates, it is difficult to isolate homogeneous species for analysis and to verify that what has been generated synthetically recapitulates what is found in the body. Much like how the static structure of a protein does not fully represent its dynamic nature nor functional state(s), A β aggregates isolated under denaturing conditions or by using methods that necessarily break apart structured oligomers cannot accurately represent their state in the brain. Furthermore, the oligomers isolated from brain tissue may not be representative of those physiologically present, and instead they may be biased due to the isolation technique. It is likely that several of the identified species (from different techniques) may have similar characteristics, but the details of techniques used to isolate and characterize these aggregates are difficult to recapitulate and validate across labs.

A wide range of A β sizes and structures have been isolated and characterized using a variety of techniques, as outlined and depicted in Table 1 and Figure 1. The techniques employed can be separated into a few categories: (i) those that can be utilized throughout the aggregation process—nuclear magnetic resonance spectroscopy (NMR), circular dichroism spectroscopy (CD), size exclusion chromatography (SEC), polyacrylamide gel electrophoresis (PAGE), and microfluidic modulation spectroscopy (MMS-IR); (ii) those used in the early stages of aggregation where LMW and toxic oligomers dominate—single molecule fluorescence microscopy, ion-mobility separation-mass spectrometry (IM-MS), and A11 oligomer antibody binding; (iii) those used in the late stages of aggregation where HMW β -sheet rich oligomers dominate—small-angle X-ray scattering (SAXS) and OC oligomer antibody binding; (iv) and those used to characterize the high molecular weight (HMW), β -sheet-rich aggregates and plaques—atomic force microscopy (AFM) and electron microscopy (EM). Thus, it is important to utilize a variety of techniques to characterize A β aggregates and to test hypotheses garnered from those experiments in various biological models to confirm their physiological relevance. An interesting example of technique bias that may mischaracterize the aggregates present in solution is the use of SDS-PAGE for analysis of A β oligomerization. Bitan and coworkers [83] have shown that SDS artificially alters the aggregation state of A β by dissociating the aggregates. This finding was independently corroborated by Hepler and coworkers [84], who obtained the same LMW (dimer-trimer) bands for oligomer and fibril preparations of A β . Thus, the use of SDS-PAGE alone is not sufficient for assessing the aggregation states of A β . Alternative methods for analysis include: native gels, SEC, and single molecule fluorescence spectroscopy for isolation and size estimation; IM-MS (combined with molecular dynamics simulations) for insights into the assembly mechanism; AFM and EM for visualization of bulk morphological features; SAXS and cryo-EM for low resolution three-dimensional arrangements; NMR spectroscopy and x-ray crystallography for atomic-level details; and the use of conformation-specific

probes for oligomer-specific recognition. Figure 2 illustrates the size ranges probed for each technique, ranging from monomeric to fibrillar assemblies.

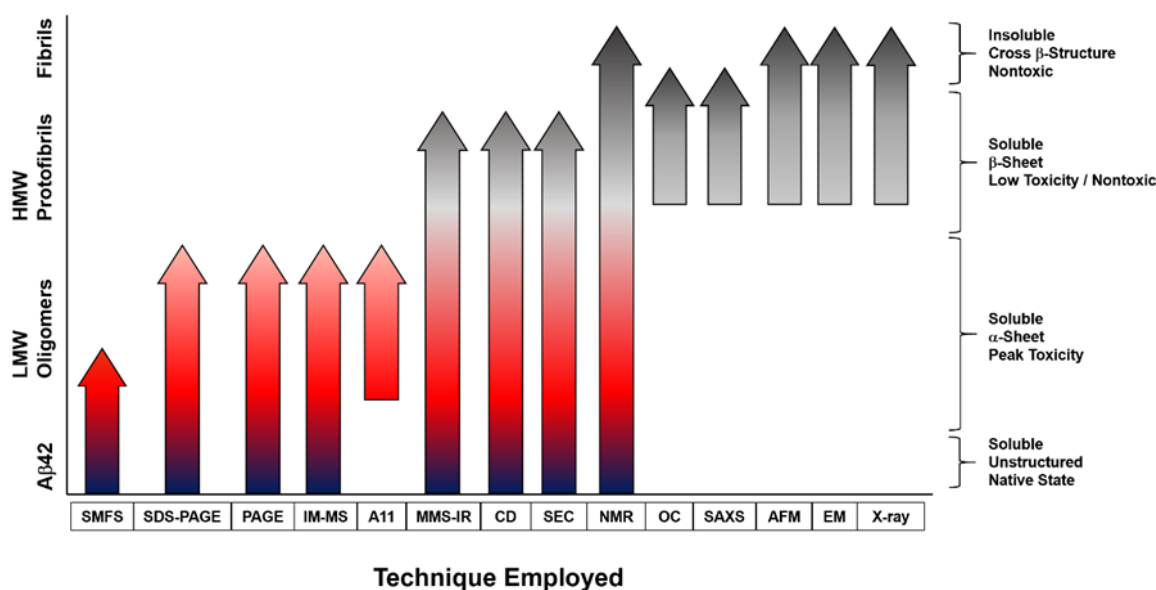


Figure 2. Isolation techniques and aggregate characteristics of toxicity and structure. Arrows span the molecular weight range that each technique can be used to characterize. Dark blue indicates largely unstructured, random coil species, red indicates assemblies that have been determined as the α -sheet-containing primary toxic aggregates, and gray indicates the HMW β -sheet aggregates and fibrils. The very LMW aggregates are soluble, unstructured, and largely nontoxic, while the intermediate 6–12 mers contain α -sheet and are the most toxic. HMW oligomers and fibrils contain β -sheet and are nontoxic.

2.1. Characterization Based on Size

The isolation and characterization of oligomeric species formed throughout aggregation is a crucial step in understanding the pathology and relevance of different aggregates in AD. Much effort has been focused on purification of samples from AD brain tissue or cultured AD-derived cells, mainly using non-denaturing PAGE and SEC [63–65]. It is important to utilize techniques that require minimal sample preparation and are non-denaturing, so that complex and heterogeneous samples can be isolated with relatively little bias. This represents a good starting point for subsequent, more specific characterizations that together provide the clearest possible window into the picture of molecular pathology.

SEC is a useful technique that characterizes distinct oligomer sizes based on a non-denaturing separation method. Recently, our lab reported [85] distinct oligomerization states of synthetic A β 42, particularly hexamer and dodecamer species by SEC. These assemblies directly correlate with early aggregation events when toxicity is highest, preceding β -sheet formation, and eventually proceed to HMW species rich in β -sheet. As described below, dodecamers (and to a lesser extent hexamers) have been implicated by a variety of different studies as the primary toxic agent(s) found in patient-derived samples, and our studies have corroborated this potential role. Importantly, we showed that oligomers are stable for extended periods of time with storage on ice at various points in the aggregation process and that isolated peaks maintain the same molecular weight (MW) upon subsequent analyses with SEC [85]. This was a critical component of our *in vitro* studies, ensuring that the oligomers are stable assemblies that can be studied reliably from the same preparations using a variety of instruments and methods.

Table 1. A β oligomers observed in different studies and by different techniques.

Species	Size & Characterizations	Isolation Technique (Source, Ie In Vitro)
SDS-stable dimers and synthetic dimers [65]	Dimers, 8–12 kDa, 3–4 nm height, no detected secondary structure	SDS-PAGE (brain derived or synthetic in vitro A β 40Ser26Cys mutant)
SDS-stable dimers/trimers [67]	Dimer/trimer, 6–12 kDa, A β 40/A β 42 with Arg5 N-term truncated species	SDS-PAGE (transfected CHO cell culture medium)
Trimers from mutant human APPV717F [64]	Trimers, 12 kDa, unstructured	SEC and PAGE (7PA2 cells)
Tetramers (A β 42 and A β 40) [80]	Tetramer, 18 kDa, ring-shaped (A β 40) or bent (A β 42), non-aggregation prone (A β 40) or aggregation prone (A β 42)	IM-MS (synthetic in vitro)
Pentamer [22]	Pentamer, compact pentagonal shape with C-termini buried	ssNMR (synthetic in vitro kinetically trapped)
Hexamer/dodecamer [85,86]	Hexamer-dodecamer, 27–56 kDa, α -sheet secondary structure, A11 positive	SEC (synthetic in vitro, human CSF)
A β *56 [63]	Dodecamer, ~56 kDa, globular, A11 positive	SEC and PAGE (Human brain isolates)
A β o [87–89]	15–20 mer, spherical vesicles 2–5 nm diameter, A11 positive	SEC (synthetic in vitro)
ADDLs [21]	Trimer–24 mer, 17 kDa tetramer major, globular, 2–5 nm height, A11-positive	Nondenaturing electrophoresis (synthetic in vitro)
ASPD (amylospheroids) [90]	32–150 mers, spheroids, 10–15 nm diameter assemblies, A11 negative	SDS-PAGE (brain derived and synthetic in vitro)
A β 42 Ellipsoids [66]	High molecular weight, ellipsoidal and annular	SAXS (Cu(II)-guided A β 42 oligomerization in vitro)
A β 40 Protofibrils [61]	High molecular weight, protofibrillar	SAXS (Cu(II)-guided A β 40 oligomerization in vitro)
HMW soluble oligomers [66]	Large, circular, 8–12 nm, form membrane-permeable pore at lipid bilayers	AFM (synthetic in vitro)

The dodecameric A β oligomer that we characterized in vitro is consistent with those previously isolated and characterized from the brains of 6-month-old transgenic mice that had begun to show symptoms of AD by Ashe and coworkers using both SEC and nondenaturing PAGE, which they refer to as A β *56 [63]. Adding the purified A β *56 directly into the brains of healthy, non-transgenic rats induces memory decline [63]. A commonly used metric for cognitive decline is the Morris water maze, which tests spatial learning and memory. This was one of the first studies to isolate and characterize a specific oligomeric species from animal brain and demonstrate a direct link between that species and toxicity in vivo. A critical component of AD research that is exploited in many studies is the fact that oligomers in the brain are stable and persist even into the late stages of disease [63–65,91,92]. This is contrary to the belief of many that these oligomers are transient and unstable, an idea likely from in vitro studies at much higher A β concentrations that drive plaque formation. Instead, the toxic oligomers are stable and can withstand processing while maintaining their structural and pathological features [20].

Another example that demonstrates the ability to isolate the stable oligomers for biophysical characterization has been described by Selkoe and coworkers, who used nondenaturing PAGE to isolate trimers from 7PA2 cells expressing the Indiana mutation of AD (affecting APP processing rather than an amino acid on A β , designated by APP_{V717F}) and showing that the trimers affect LTP more strongly than other HMW aggregates [64]. Additionally, LTP decreases when dimers directly isolated from the brains of AD patients are added to the brains of healthy wild type rats [65]. This, however, poses the question of whether these LMW species are directly responsible for toxicity, or if they rapidly aggregate

and form the putative species to induce toxicity. Nonetheless, Selkoe and coworkers present the argument that soluble, LMW oligomers are on-pathway aggregates that directly affect cell death and LTP [65].

To combat the difficulty of determining exact size distributions of A β 40 oligomers, single molecule fluorescence spectroscopy was recently employed [93]. This method quantitatively infers the number of monomer subunits in an assembly by counting individual fluorophore-labeled A β peptides. This technique works at more physiologically relevant A β concentrations and is performed at the single molecule level so that it gives insights as to population heterogeneity rather than bulk characteristics. Single molecule fluorescence spectroscopy determines the heterogeneity implicit in a given sample rather than heterogeneity induced by preparation protocols, and it has the potential to flesh out individual assemblies responsible for toxicity. However, the question remains as to the influence of sample preparation, particularly for the more 'sticky' A β 42 peptide, and the effect of fluorescent probes on which species are formed during aggregation and whether these protocols recapitulate what is present in vivo.

SAXS is another technique employed to monitor A β oligomerization [94]. SAXS has much lower spatial resolution; however, its temporal resolution is much better when combined with in-line rapid mixing. This combination gives critical information as to the early events in aggregation, and the results can give insights into the diameter, molecular weight, and polydispersity of oligomeric species [94]. Ryan and coworkers used SAXS to investigate the influence of Cu(II) on A β oligomerization, as Cu(II) is proposed to play a role in AD pathogenesis. Interestingly, they found that A β 42 aggregates form ellipsoids in the presence of Cu(II), which recapitulates other widely reported annular structures, whereas A β 40 forms protofibrillar structures [66,80,94,95]. SAXS presents a unique opportunity to monitor the early events in the aggregation of A β that spur the eventual fibrillization. Characterizing the populations of aggregates formed throughout disease progression is the first critical step in elucidating a mechanism for AD. The next step, then, is to ask how these discrete assemblies induce toxicity, and the structural basis for the mechanism.

2.2. Conformational Insights Provide a Basis for Toxicity

Purification and characterization of A β oligomers are essential in determining the specific assemblies in the heterogeneous distribution responsible for toxicity. Previously, it has been stated that unstructured oligomers are regarded as nontoxic [69,96,97], whereas structured oligomers are much more likely to be toxic [61,66,80,81,85]. Interestingly, Kaye and coworkers developed a polyclonal antibody (A11) that cross-reacts with a wide range of amyloid peptides and proteins in the oligomer state, independent of sequence and native starting structure [63,87–89]. This discovery implicates a common structural motif formed during oligomerization that is likely associated with the toxic state, as this antibody was able to mitigate toxicity of each of the oligomeric species. It is necessary, then, to study the structural characteristics of A β oligomers throughout aggregation to better understand the process and which features contribute to the toxicity mechanism. Several labs have undertaken this biophysical approach to studying AD, in a variety of different ways, as discussed below.

Our lab has recently presented evidence for the α -sheet hypothesis in A β 42 aggregation and the structure of the toxic oligomers [85]. The α -sheet structure is a unique, nonstandard secondary structure with hydrogen bonding patterns and spectroscopic characteristics distinct from those of α -helix, β -sheet, or random coil [85,98–105]. The α -sheet structure was discovered in molecular dynamics simulations (MD) of amyloidogenic proteins as they unfolded and misfolded under amyloidogenic conditions. This unique conformation was predicted to be a key component of the toxic soluble oligomers and was verified as the dominant secondary structure formed during the lag phase of aggregation in vitro prior to formation of β -sheet structure [85,101–104]. Based on the α -sheet structure seen in the MD simulations, we designed complementary de novo, nontoxic, synthetic peptides. We used alternating L- and D-amino acids to engineer a stable form of α -sheet that does not

aggregate and is recognized by the A11 antibody, consistent with the α -sheet structure being present in amyloid oligomers [85,101–105]. Furthermore, these de novo α -sheet peptides mitigate toxicity in cell culture, and specifically bind and neutralize soluble toxic oligomeric aggregates in both an ex vivo and in vivo transgenic AD mouse model, and in vivo AD *C. elegans* models [85]. In addition, the designed α -sheet peptides are being used to detect toxic oligomers in A β 42 samples in PBS [85], spiked into plasma and CSF, and human CSF and plasma from AD patients [86]. The α -sheet hypothesis poses a new avenue for the conformation-specific detection of toxic aggregates in AD, and the use of sophisticated techniques to determine the conformation of aggregates may help shed light on this and other proposed mechanisms [105]. Notably, the discovery of α -sheet structure is the direct result of years of working out methods to simulate protein unfolding/folding in combination with validation via protein engineering experiments [106,107].

Regarding the conformational characterization of aggregates, IM-MS is a promising technique for interrogating the structure of oligomeric assemblies, and it has been used to suggest that A β 40 and A β 42 form distinct conformations [80,81]. For example, A β 40 tetramers form a compact ring-shaped structure, making it more difficult to form further contacts. Alternatively, A β 42 tetramers prefer a bent structure with subunits at either end that easily facilitate the addition of subsequent species. This technique showed that A β 40 oligomers remain smaller, while the A β 42 tetramers further assemble into large donut-shaped dodecamers, which could seed and accelerate protofibril formation and elongation [80,81]. As the dodecamers (A β *56) have been shown to specifically cause memory deficits [63], these IM-MS studies shed light on the higher order structure behind the biologically relevant A β 42 toxic isoform.

AFM and EM are also useful techniques to gain insights as to the structural topology of specific aggregates. These two techniques have been used to understand the morphologies of oligomers and fibrils that form on the micrometer or nanometer scale [61,66]. AFM in particular has been used to probe the morphology of oligomers at lipid bilayers. One study demonstrated that A β 42 oligomers assemble into circular aggregates on the order of 8–12 nm that interact with the lipid bilayers to form a membrane-permeable pore [66]. This mechanism of toxicity is specifically implicated to trigger flow through the cell membrane leading to cell death and provides an explanation for the toxic nature of the more structured A β oligomers over the amorphous aggregates [66,108].

2.3. Atomic Details of Toxic and Nontoxic Oligomer Conformations

The intrinsic heterogeneity and dynamic nature of A β oligomers have made high-resolution structural analysis of A β oligomers very difficult. Despite this, 2D NMR spectroscopy [22,109] and X-ray crystallography [110] have been used to probe the atomic details of kinetically trapped oligomers. Ishii and coworkers [109] proposed that despite the size heterogeneity of prefibrillar A β 40 oligomers, they maintain parallel β -strand structure similar to amyloid fibrils. Incubating the solution at 4 °C and flash freezing them in liquid nitrogen allowed them to quickly collect 2D solid state NMR spectra at 15 °C [109]. Though the oligomers produce less overall Nuclear Overhauser Effect crosspeaks (NOEs) and form less contacts than the fibrils, they claim that the similar spectral characteristics and connectivities displayed by key amino acids throughout the peptide indicate that the underlying structures must be related despite major morphological differences. Importantly, they focus on 650 kDa, non-A11 reactive spherical aggregates that form just prior to fibril deposition, indicating that they are likely the late stage β -sheet protofibrils that seed fibril formation and are not directly associated with the physiological toxicity mechanism.

Some labs have turned to the use of non-physiological A β peptide fragments and protein engineering techniques to explore potential sequence-mediated aggregation mechanisms. Pham and coworkers utilized X-ray crystallography to determine a crystal structure of oligomers formed by a core A β segment (containing residues 15–23) and demonstrate how this region generates a wide array of oligomer assemblies, possibly due in part to the missing residues [110]. Once the discovery of self-propagating molecular-level poly-

morphism in A β fibrils was established, protocols for preparing relatively homogenous samples were developed [90,111] and structural models for A β 40 polymorphs were produced from solid state NMR data. Smith and coworkers [22] also used solution-based NMR to acquire a number of NOEs to aid in the development of pentamer and fibril models at low temperature and low salt conditions. Unfortunately, however, atomic-level details of full-length LMW toxic A β oligomers under physiological conditions have yet to be elucidated due to the lack of a sufficient number of experimental observables for generation of atomic models.

2.4. Toward Characterization of Brain-Derived Oligomers

Several labs have shown that brain-derived soluble A β species can be extracted from AD patients using saline buffers without detergents [16,112]. The extracted samples can then be fractionated with nondenaturing SEC to isolate individual assemblies for discrete characterizations [16,112]. Using such methods, O’Nuallain and coworkers demonstrated that synaptotoxicity correlates with soluble A β oligomers ranging from dimers-hexamers [16,112]. Shankar and coworkers used SDS-PAGE to characterize dimeric A β present in AD tissue and suggest that this dimer is the minimal toxic component in vivo, but due to recognized artifacts with this technique, questions remain as to whether the toxicity is directly caused by these dimers [65]. Dissociation into low MW species by using SDS-PAGE and rapid aggregation of the putative dimers into larger aggregates might provide an alternate explanation. In a later study, these authors found that synthetically prepared dimers—used as a model for the brain-derived species—rapidly aggregate into metastable protofibrils, which suggests a much more complex process is likely at play [113]. Larger A β oligomers, >100 kDa and A11-negative, with spherical structures roughly 10 nm across have also been isolated from AD-affected brain tissue and been proposed as the likely toxic agents, further confounding the study of brain-derived oligomers [114,115]. These assertions are in opposition to Ashe and coworkers who have reproducibly isolated the A β *56 oligomer from AD brains, indicating that the LMW oligomers responsible for synaptotoxicity are present, despite not being a primary component in the pathological characterizations (which rely on plaque burden) [63]. Furthermore, Ashe and coworkers found that the larger MW oligomers are OC-positive (OC is an antibody that specifically binds the fibril plaques, Figures 1 and 2) and are not toxic. Nonetheless, that this range of soluble oligomers is stable enough to be isolated from diseased human brains is again critical to the study of AD, indicating that oligomers are persistent in both the beginning and late stages of disease where the primary pathology is plaque burden.

3. Mechanisms of Toxicity by Oligomers

In recent years, the view that the low molecular weight, soluble oligomers are the primary toxic agents has become more widely accepted. Furthermore, neuronal damage begins to occur 10–20 years before presentation of the symptoms in AD patients, and amyloid plaques are late-stage pathological indicators of the disease [12–66]. Due to the heterogeneous and dynamic nature of the A β oligomers, the mechanisms by which these aggregates carry out their toxic effects and initiate neuronal death is difficult to determine; however, increasing information about the mechanism of action of these oligomers is becoming available (Figure 3).

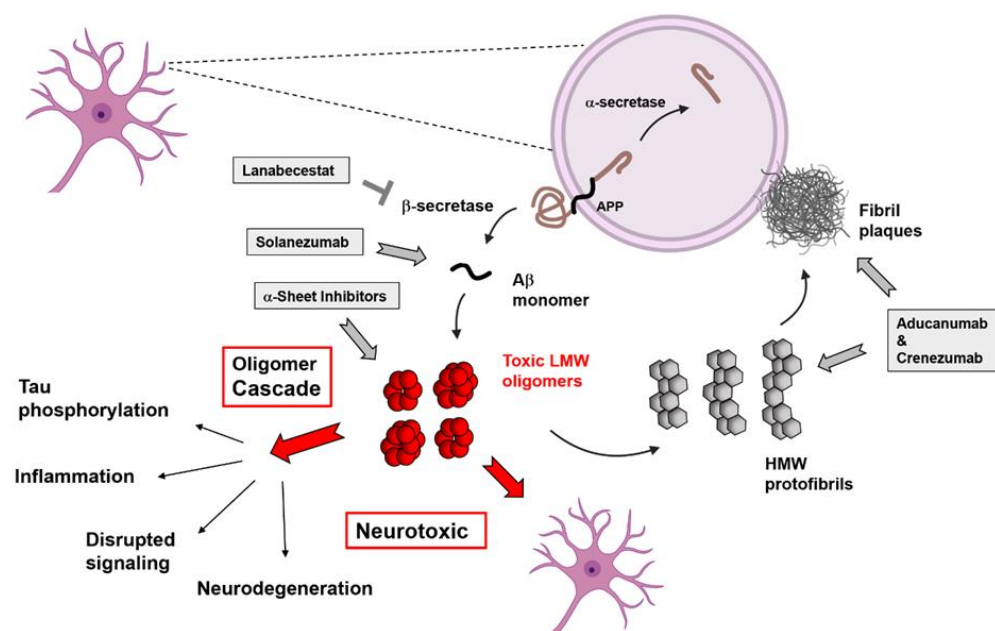


Figure 3. Drug candidates and their associated targets. Processing of APP by β -secretase produces monomeric, native A β . A β undergoes a misfolding event that eventually produces LMW oligomers that are neurotoxic and initiate the oligomer toxicity cascade. These eventually progress to HMW protofibrils that finally deposit as fibril plaques at the neuronal membrane. LanabecestatTM is a BACE inhibitor that reduces processing of APP to produce A β monomer, while SolanezumabTM is a monomer (sequence) specific drug. CrenezumabTM specifically targets the β -sheet rich HMW protofibrils and fibrillar plaque deposits, while AducanumabTM specifically targets the β -sheet fibril plaques.

3.1. Membrane Interactions Foster A β Toxicity

It has been proposed that A β oligomers affect neuronal membranes by a number of different mechanisms. Synapse loss is an indication of AD and greatly contributes to the cognitive deterioration of patients, which is often preceded by attenuation of LTP, lowered synaptic plasticity, and decline of spine density [116]. These neurological features have been known for years, but recent studies have shed light on the mechanistic details.

Some studies have shown that A β oligomers bind to the neuronal membrane, causing local perturbations that damage membrane integrity [108,117,118]. Specifically, SDS-PAGE-isolated A β dimers accumulate at lipid rafts in the brains of transgenic Tg2576 mice, which overexpress the Swedish mutation (APP_{KM670/671NL}) causing increased production of A β 40 and A β 42 [118]. Importantly, the dense localization of A β oligomers at lipid rafts increases the local concentration of A β and might help to seed aggregation and plaque formation, which is not found in most cases at physiologically relevant concentrations. This may help to explain why the A β monomers show little inclination to aggregate into the toxic oligomers at such low concentrations in healthy brains, but under certain circumstances that promote localization they misfold, aggregate, and form the toxic species. Furthermore, Selkoe and coworkers confirmed the observation that SDS-dimers interact with lipid membranes much more rapidly than their monomeric counterparts [118]. Upon immunoprecipitation, the dimers associate with GM1-gangliosides in the lipid rafts. These sites are hubs for signal transduction, and the density of oligomers in these regions necessitates the oligomer effect on signal propagation, thus aiding in memory decline. This result is further supported by the correlation of A β dimer localization at lipid rafts in 6-month-old Tg2576 mice with memory impairment symptoms [117]. Disregarding the assertion of dimer-specific influence here due to the issues associated with SDS-isolation, the fact that the misfolded and aggregated forms of A β —and not the monomeric form—localize at neuronal membranes indicates that misfolding and localization play important roles in the pathogenesis in AD.

Some studies show a more active form of toxicity exacted by A β oligomers at the membrane [119]. The formation of annular structures ranging from 40–170 kDa (from combined SDS-PAGE and AFM studies) insert themselves at the neuronal membrane to initiate dysregulation of efflux/influx through the creation of membrane pores [108,119,120]. The perforation of neuronal membranes has been attributed to the antiparallel β -sheet conformation of late-stage oligomers and fibrils, which disrupt cellular systems [120]. This penetration leaves large channels where passive diffusion of small molecules takes place, thus dysregulating the intracellular and extracellular environment. Alternatively, some researchers propose that the pore formation is instead due to the formation of α -helical structures linked to a repeat motif of GxxxG in the A β peptide [121,122]. It is proposed that this motif may facilitate the formation of α -helical structures that form pores at the membrane and disrupt molecular flux. Along these lines, as support for the possibility that the pores may contain α -sheet, we note that the potassium channel contains four α -strands with the aligned main chain carbonyl oxygens pointing toward the center of the channel to facilitate ion flow [100,123].

3.2. Intracellular Effects of A β Oligomers

Receptor-mediated toxicity may present an even more guided mechanism of A β oligomer toxicity through endocytosis at specific sites on the membrane. Several studies have demonstrated the binding of A β oligomers to receptors on the neuronal membrane, leading to endocytosis of oligomers into the intracellular space and initiating damage that affects neuronal signaling [124–126]. Treatment of astrocytes with oligomeric aggregates of A β specifically increases glutamate release, as detected with a FRET-based glutamate sensor; however, this effect is not observed following treatment with A β monomers [124]. This glutamate response is calcium-dependent and initiated by the binding of A β trimers (isolated by SEC) to the α 7 nicotinic acetylcholine receptors of astrocytes, which is a calcium permeable ion channel [124]. The effect on synapse signaling is due largely to the overactivation of extrasynaptic N-methyl-D-aspartate receptors (eNMDAR) on hippocampal neurons following the glutamate increase. Additionally, these studies show that A β oligomer-receptor complexes promote internalization through endocytosis that triggers damage specifically to the intracellular compartments [126]. This complexation affects cellular signaling; for example, the activation of eNMDARs by SEC-isolated trimers increases calcium flux, which triggers nitric oxide synthase to increase NO production, which then leads to apoptosis [124]. Increased NO levels also affect synaptic spine density, where a loss often results in degeneration of neuronal synapses and thus connectivity. These complexes activate specific kinases that decrease NMDAR density and cause dendritic spine loss. In this case, A β oligomer toxicity is carried out by both receptor- and spine density-mediated mechanisms, purportedly due to the misfolded prion-like activity of the oligomer species [124,126].

Extracellular A β can interact with membranes and receptors, but it can also be internalized into neurons by endocytosis. Additionally, A β accumulates intracellularly where APP is present in the membrane [127–129]. The mitochondria, endoplasmic reticulum (ER), *trans*-Golgi network, endosomes, autophagosomes, and lysosomes are all implicated as potential sites for A β generation and aggregation. Intracellular A β oligomers can enact cellular damage and initiate cell death through elevated ER stress, mitochondrial damage, calcium ion dysregulation, and apoptosis [127–129].

Umeda and coworkers used an A $\beta_{E22\Delta}$ (APP_{E693 Δ}) transgenic mouse line to express mutant A β (termed the Osaka mutant) that forms soluble oligomers but does not form fibrils [27,31,32,127]. They demonstrated that aggregates (of an undetermined size) colocalize at cellular organelles in neurons, and these mice have elevated levels of markers indicative of heightened ER stress [127]. This stress is specifically due to the intracellular accumulation of A β oligomers and activated by phospholipase C signaling, which initiates release of calcium from the ER into the cytosol [128]. Cell viability specifically decreases and caspase 3 (an apoptotic initiator) is activated in response to this dysregulation [127,129]. The Osaka

mutant is a very unique case of AD, but importantly portrays the essential role that soluble oligomers play in the toxicity mechanism, even in the absence of amyloid fibrils.

This brief summary of the vast number of alternative pathways of A β oligomer-induced toxicity demonstrates that no single mechanism explains all aspects of toxicity in AD. The process is multifaceted and may involve a myriad of interconnected processes and signaling pathways to initiate the neurodegenerative decline characteristic of AD. Importantly, however, there is recent evidence from several groups for a more generic type of toxicity. Wogulis et al. have shown that the ongoing self-assembly of A β 42 into fibrils from soluble monomers and oligomers is, by itself, sufficient to cause cell impairment and death [130]. Additionally, synthetic α -sheet peptides designed to specifically target the toxic species neutralize the toxicity and prevent further aggregation [85]. Thus, the dynamic aggregation-based model of toxicity is compatible with most observations in the literature; however, an exact mechanism has yet to be elucidated, but a more nuanced mechanism involving lipids facilitating membrane disruption is intriguing [131].

4. A β -Based AD Treatments in Clinical Trials

There have been a wide range of small molecules and antibodies that have entered clinical trials in recent decades for the treatment of Alzheimer's disease. Trials include compounds from large companies such as Biogen, Roche, Genentech, and many more, and despite the abundance of funding and manpower dedicated to drug development, there has been little clinical success [132]. The clinical trials have taken three main approaches: (1) disrupting the fibrillar form of A β by binding to and breaking up plaques; (2) sequestering the monomeric form of A β through sequence-specific binding; or (3) halting the production of A β by inhibiting the β -secretase enzyme that clips APP to give rise to A β (BACE inhibitors) [132]. While there has been much discussion of enrolling patients earlier to obtain better outcomes, it would be inherently difficult to obtain significant clinical efficacy with any of these approaches. Targeting fibrils is not only an intervention that takes place too late in the disease, but it has also been shown to increase the level of soluble oligomers in the brain, thereby increasing toxicity rather than halting disease progression [82,132]. Similarly, targeting the monomer directly through sequence recognition or with BACE inhibitors would remove the physiologically necessary form of A β from the brain, thereby disrupting processes that promote normal function, not to mention affecting the processing of other peptides [5–10,132]. Recently several companies have claimed that their compounds target the soluble oligomers formed in the early stages of disease, but further analysis of the antibodies and small molecules used indicates that they are targeting late-stage β -sheet rich protofibrils, fibril plaque deposits, or they indiscriminately bind different A β conformers. We outline a few of the A β targeting approaches below.

AducanumabTM is a monoclonal antibody that preferentially binds to the protofibrils and plaques formed in the late stages of disease (Figure 3) [132–134]. Aducanumab was developed using an immunotherapeutic approach wherein human B-cell clones were exposed to aggregated A β and screened for reactivity [133]. The resultant library went through molecular cloning, sequencing, and recombinant expression and eventually resulted in the monoclonal antibody Aducanumab, which selectively reacts with both soluble oligomers and insoluble fibrils [133]. The cross-reactivity of the monoclonal antibody for soluble and insoluble forms of aggregated A β indicates that the recognition motif is likely the β -sheet structure shared by late-stage protofibrillar HMW aggregates and the fibril plaque deposits. Due to the binding preference of this antibody, measures of efficacy were necessarily contingent upon initial plaque presence in a variety of animal and human cohorts and was verified using amyloid PET imaging (which uses a plaque-specific radiotracer to measure amyloid burden). Thus, enrolled patients were likely in the mid-to-late stages of the disease when they were undergoing treatment. The hope was that Aducanumab would be an effective intervention in the early stages of the disease [134], but it appears to be targeting a late-stage indicator of disease progression [132,133]. Aducanumab (marketed

as Aduhelm) has recently been approved for use by the FDA but is undergoing continued scrutiny due to a lack of consistent data in clinical trials.

SolanezumabTM is a monomer-specific monoclonal antibody probe that has been discussed as a potential early-stage therapeutic option for AD patients in the mild cognitive impairment (MCI) stage (Figure 3) [132,135,136]. The parent monoclonal antibody for Solanezumab (m266) was generated in A/J mice using a synthetic A β 13-28 peptide (HHQKLVFFAEDVGSNGGC) where several positive clones were identified that all selectively bound to A β 1-28, but not A β 1-16 nor A β 17-28, suggesting specificity for residues 13-28 of A β [135]. Early characterization showed that this antibody was unable to bind to A β aggregates, but completely prevented A β 42 fibrillization in vitro by binding and sequestering the monomeric peptide [135]. Thus, Solanezumab is considered the AD paradigm of a sequence-based antibody that selectively binds the free, not solely the aggregated, form of A β . Based on past clinical trials and the scientific literature surrounding A β functions and toxicity mechanisms, sequence-based interventions for AD will not halt nor prevent disease progression [132]. In fact, monomer-targeting compounds have been associated with various side effects, likely due to removal of the physiologically active form of the peptide from the brain [132]. This may produce positive results in the short-term, which is generally measured in animal models and early trials; however, the long-term rehabilitative effects are much more difficult to determine.

CrenezumabTM is a monoclonal antibody that has been used in several AD clinical trials, promoted as a truly oligomer-specific marker that binds to the LMW, soluble oligomers formed in the early stages of AD (Figure 3) [132,137–139]. Crenezumab was generated by immunizing mice with an A β 1-15 peptide antigen and screening for antibodies that bound several different species of A β (including monomer) and inhibited A β 42 assembly into small cytotoxic aggregates [139]. Early clinical studies indicated that Crenezumab specifically reduces A β oligomers present in CSF after treatment in humans and a variety of different animals [137–139]. Interestingly, however, the antibody specifically binds to protofibrillar A β and mature amyloid plaques, indicating that the conformational specificity is for the β -sheet structure formed in the late stages of disease [138].

Finally, LanabecestatTM is one of many examples of BACE inhibitor therapeutics tested for AD treatment (Figure 3) [132,140]. Lanabecestat works by inhibiting the β -secretase enzyme from clipping at the β -site of APP, thus mitigating the production of A β monomers in the brain. Many labs have shown the efficacy of this approach in vitro, animal, and early clinical models [132,140]. Removal of A β prevents formation of the toxic oligomers, protofibrils, and plaques. The Lanabecestat clinical trial was eventually stopped under futility analysis, unlike similar BACE inhibitors VerubecestatTM and AtabecestatTM, which were halted due to side effects [131]. Even though Lanabecestat was well tolerated, neither of the two doses tested was effective at shifting the primary or secondary outcomes from placebo [132]. Targeting BACE is an enticing strategy for treating AD, as mitigating the production of more A β monomers can halt further oligomer/fibril formation; however, as most patients will be treated only after showing symptoms, oligomers and fibrils are largely already present. Moreover, there are concerns about the removal of A β monomers given their many biological functions [5–10], as well as other peptides processed by the inhibitor secretases [3,12,15–17,74,75,132,140].

5. Conclusions and Outlook

AD is an amyloid disease characterized by the aggregation of the A β peptide monomer into a heterogeneous distribution of soluble, toxic oligomers, followed by relatively nontoxic protofibrils, and eventually results in the characteristic nontoxic cross- β pleated sheet fibrils. The toxic intermediates are dynamic in nature, making the study of their size, structure, and the detailed mechanism of toxicity difficult to characterize. The fibrils are nontoxic, but they can act as a reservoir of soluble oligomers by its fragmentation and secondary nucleation. Recent advances in biophysical and biochemical methods have provided more insights into the structural characteristics of the oligomers formed throughout aggregation. Isolation

and size estimation using PAGE and SEC have led to informed studies of the effects that different size aggregates have on neuronal function. However, it must be noted that while the isolation of oligomers helps us to characterize the process, the effect of isolation is not fully known, and, in particular, as discussed above, there are known artifacts with PAGE methods. Assembly pathways have been investigated with the aid of IM-MS experiments and MD simulations. Bulk features of morphology and topology have been directly visualized with techniques like AFM and EM, while more detailed information has been obtained with NMR and X-ray crystallography. Importantly, conformation-specific probes have been developed to investigate and interfere with specifically assembled structures that are associated with toxicity.

Along with advances in oligomer characterization, many toxicity mechanisms governed by A β oligomers have been proposed and investigated. These mechanisms range from extracellular interactions with membranes or receptors, intracellular accumulation disrupting normal function, and cell-to-cell transmission of toxic aggregates. Researchers are shifting their focus now to the relationship between oligomer conformations and toxicity and using this information to design probes that can intervene in the early stages of disease progression for diagnosis and treatment. In this regard, it is critical for researchers to carefully report the details of their oligomer preparation protocols and experimental conditions to compare what is done in different labs to help facilitate progress in the field and ensure reproducibility.

The unfortunate failures of past AD clinical trials have been taken as a failure of the amyloid cascade hypothesis, and this in turn has spurred efforts focused on other targets. While expanding the scope to investigate other targets is overall beneficial for the field, we and others argue that it is not a failure of the amyloid cascade hypothesis *per se*. Instead, these failures illustrate the importance of clearly distinguishing and targeting specific A β conformational species, rather than collectively lumping together states that are conformationally and physically distinct into a catch-all amyloid state. To avoid confusion, it is best to reserve the term amyloid for true amyloid fibrils and plaques. Given these and other factors that have come to light in the last years [141,142], the amyloid hypothesis is better denoted as the oligomer cascade hypothesis. This highlights the importance of the early-stage toxic oligomeric species as well as the dire need for a shift in AD diagnosis and treatment toward an oligomer-based approach. The studies above have outlined the latency for AD symptoms years after neuronal damage has taken place. An associated component in the refinement of therapeutic efforts is that AD diagnostics must detect individuals at risk earlier, prior to the development of the current late-stage disease markers. In this case, new therapeutics could then be tested for their ability to mitigate disease progression, rather than trying to reverse neuronal damage that has taken place over 10–20 years. On a more sobering note, AD is incredibly complex and when it appears that we have a handle on the many effects of toxic oligomers, taking a narrow example, and then new neuron-specific deleterious activities are discovered and, as ever, the devil is in the details [143].

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Abbreviations

Abbreviation	Term
AD	Alzheimer's Disease
Ab	Amyloid beta peptide
APP	Amyloid Precursor Protein
AFM	Atomic Force Microscopy
BACE Inhibitor	Beta-secretase inhibitor
CD	Circular Dichroism Spectroscopy
EM	Electron Microscopy
ER	Endoplasmic Reticulum
eNMDAR	Extrasynaptic N-methyl-D-aspartate Receptors
FTIR	Fourier Transform IR
Forster Resonance energy transfer	FRET
HMW	High Molecular Weight
IM-MS	Ion-Mobility Separation-Mass Spectrometry
LTP	Long Term Potentiation
LMW	Low Molecular Weight
MMS-IR	Microfluidic Modulation Spectroscopy
MCI	Mild Cognitive Impairment
MD	Molecular Dynamics
NMR	Nuclear Magnetic Resonance Spectroscopy
NOEs	Nuclear Overhauser Effect Cross Peaks
PAGE	Polyacrylamide Gel Electrophoresis
PET	Positron Emission Tomography
SEC	Size Exclusion Chromatography
SAXS	Small-angle X-ray Scattering
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

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