Monitoring the Aggregation of Amyloid-β in the Presence of Polyphenols with Emphasis on Curcumin

Valerie Winkler, Brittany Hagenhoff
Truman State University, Kirksville, MO 63501

The aggregation of amyloid-β (Aβ) into fibrils forms the plaques implicated in the pathology of Alzheimer’s disease, an aggressive cognitive disorder that impairs function of the cerebral cortex. Therefore, discovery of a mechanism by which fibril disaggregation occurs is of vital importance to the medical community. Here, the current knowledge of and methods to measure Aβ aggregation are reviewed, specifically focusing on the formulation of fluorescence assays as well as a review of the use of polyphenolic compounds as disaggregation agents. One such polyphenol is curcumin, a curcuminoid derived from Curcuma longa that has seen some effective results for reducing Aβ aggregation; however, potential intermolecular interaction complications should be considered in analysis, especially in the popular emission assay studies with Thioflavin T as a fluorescent probe. Overall, future work to determine the applicability of curcumin, as well as other polyphenols, on the prevention or disassembly of Aβ fibrils must account for intermolecular interactions by experiment and the employment of computational means to ascertain an accurate degree of potential therapeutic effect.

Abbreviations: Aβ – Amyloid-β; APP – Amyloid Precursor Protein; ROS – Reactive Oxidative Species; AFM – Atomic Force Microscopy; ThT – Thioflavin T

Keywords: Amyloid-β; Alzheimer’s; Curcumin; Polyphenol; Thioflavin T; Fluorescence

Introduction

Affecting almost 10% of the population over 65 years of age in the United States, Alzheimer’s disease is the sixth leading cause of death in this country (Alzheimer’s, 2020). The original description as “a peculiar disease of the cerebral cortex” was the title of the published paper in 1907 by Alois Alzheimer which primarily characterized the affliction by the loss of neurons, neuropsychiatric symptoms, and the buildup of plaques that, along with neurofibrillary tangles composed of hyperphosphorylated tau proteins, impede neurological functioning (Alzheimer, 1907; Heneka et al., 2010; Lyketsos et al., 2011; Jakob-Roetne and Jacobsen, 2009; Ballatore et al., 2007; Buée et al., 2000; Iqbal et al., 2009; Savelieff et al., 2013). Currently, there is no cure for Alzheimer’s disease; thus, it is vital to identify methods to slow down, prevent, or reverse the progression of this debilitating, chronic illness. The current drugs that have been approved by the Food and Drug Administration for use in symptomatic treatment are used to increase the amount of neurotransmitters and/or block neuron overstimulation; these are used only in the context of symptom management and do not prevent disease progression (Alzheimer’s Association, 2020).

One method postulated to accomplish deceleration of disease progression is to target the rate of plaque formation. These plaques are primarily composed of fibrils formed by the aggregation of the amyloid-β (Aβ) peptide, discovered by Glenner and Wong and formed the foundation for the “amyloid hypothesis” that has driven the community focus on dissembling these fibrillar plaques (Glenner and Wong, 1984). To glean more understanding of the
formation of these plaques, plenty of research has been accomplished to ascertain the mechanism of assembly and three-dimensional structure of the Aβ fibrils. Additionally, many methods for the quantitative determination of Aβ concentration have been proposed and published, though some are more favored to certain sets of conditions than others, and thus optimization is necessary depending on the exact nature of the experiment at hand. One particular method that has been under scrutiny is the Thioflavin T (ThT) fluorescence assay, an in vitro monitoring technique of quantification for Aβ fibrils. This method is considered controversial in some cases, specifically for studying the effects of polyphenols on the rate of Aβ aggregation over time due to potential impact of the polyphenol’s inclusion on the inflation or quenching of the ThT fluorescence signal intensity.

Polyphenols are a class of compounds characterized by the presence of multiple aromatic rings with hydroxyl substituents and are a topic of investigation with regard to destabilizing Aβ fibrils. Many compounds have been observed to promote Aβ fibril destabilization, such as quercetin, resveratrol, and (-)-epicatechin, though other polyphenols studied have been proven effective (Wang et al., 2011; Huebbe et al., 2010; Heo and Lee, 2005). Of particular interest is elucidating the mechanism by which fibril destabilization by polyphenols occurs, which once determined may open up more therapeutic options for attaining this goal. This requires finding and defining the primary region of interaction. The polyphenol curcumin, belonging to the subset of curcuminoid polyphenols, has both acclaimed praise and come under fire in various reports of fibril destabilization efficacy. Therefore, a particular emphasis here is to elucidate why this has been the case and to determine curcumin’s efficacy as an Aβ fibril destabilizer.

The purpose of this review is to (a) explain current literature reporting on the mechanism of Aβ fibril formation and analyze current methods of monitoring Aβ aggregation, (b) look at the current studies of the efficacy of polyphenolic compounds as fibril destabilization and/or preventative agents, and (c) initiate an in-depth investigation into the potential of curcumin for use in disaggregation and/or prevention of Aβ fibrils, specifically with regards to various fragments of the Aβ peptide.

**Amyloid-β**

**Peptide Structure**

Knowledge of Aβ fibrilization is vital for understanding how the plaques that impede neuronal functioning and communication are formed, and this begins with information on the monomeric Aβ structure and its synthetic pathway. The primary structure of the Aβ peptide is 38-43 amino acid residues, with 1-40 and 1-42 being the focus of most research (Glenner and Wong, 1984; Jakob-Roetne and Jacobsen, 2009). The residue chain itself has some interesting features: the first section of 28 residues is relatively polar in nature, containing several acidic and basic amino acids, whereas the remainder of the peptide is quite nonpolar, implying hydrophobicity of that portion (Figure 1).

![Amino acid sequence for the Aβ peptide residues 1-43.](image)

Aβ is produced from the amyloid β/A4 precursor protein (APP). In its prominent neuronal form, APP has 695 residues in its primary sequence, with common alternative splices that result in a length of 751 or 770 residues due to exons containing 19 or 56 amino acids inserted at residue 289 (Glenner and Wong, 1984; Ponte et al., 1988; Selkoe, 1990). Cleavage may occur by a non-amyloidogenic pathway where α-secretase cleaves APP within the Aβ transmembrane sequence or by an amyloidogenic pathway where β- and γ-secretase cleave APP at the N- and C- termini, respectively, with the multiple γ-secretase cleavage sites resulting in the Aβ length variations observed (Mills and Reiner, 1999; Nalivaeva and Turner, 2013). The non-amyloidogenic pathway is generally favored, but the amyloidogenic pathway does occur (Nalivaeva and Turner, 2013; Cheignon et al., 2018). While APP is known to contain the Aβ
peptide, the purpose of APP is not fully elucidated, although this transmembrane glycoprotein is ubiquitous, and APP may have a function as a type of cell surface receptor (Kang et al., 1987). However, there is evidence to suggest that APP is utilized in choroid plexus development in embryological stages of sheep and, by extracellular excretion, has a role in neurite extension (Nalivaeva and Turner, 2013; Schubert and Otero, 1994; Tu et al., 1990). Either way, it is known that the production of Aβ peptides from APP and the fibrillar formation process are implicated in the pathology of Alzheimer’s disease.

**Role of Aβ in Oxidative Stress**

Oxidation-reduction reactions are plentiful in biological environments and are directly involved in the Aβ fibril formation process that results in the buildup of the interneuronal plaques present in the pathology of Alzheimer’s disease. One example would be the general reduction of oxygen (Eqn.1):

\[ O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \]  

(1)

which is the normal path of occurrence as oxygen is the electron acceptor in the final stage of cellular respiration. Partial oxidation may also occur (Eqns. 2-4):

\[ O_2 + e^- \rightarrow O_2^- \]  
\[ O_2^- + e^- + 2H^+ \rightarrow H_2O_2 \]  
\[ H_2O_2 + e^- \rightarrow H^+ + O_2^- + H^+ \]  

(2-4)

and this may be problematic, as the radicals are reactive oxygen species (ROS) that are unstable and tend to snatch electrons from other molecules to obtain electronic stability. Some ROS are utilized in signaling processes and are thus expected to be present in the extracellular environment. However, since the structure of proteins is inherently tied to their function, ionizing or radicalizing an amino acid may disrupt proper protein functioning. As proteins, including Aβ, are readily abundant in biological systems, plenty of damage can be caused if the concentration of ROS is too high in a particular location (Nalivaeva and Turner, 2013). By this principle, the amount of oxidative stress that a system is under may be tied to the presence of ROS.

In Alzheimer’s disease, oxidative stress in the brain has been linked to the early stages of disease onset (Butterfield et al., 2010). Specifically, the methionine at position 35 has been implicated as a likely culprit for the increase in oxidative stress, as replacement with cysteine in *C. elegans* resulted in oxidative stress attenuation and in *vivo* rodent studies observed that the Aβ 1-16 mutant did not produce any oxidative stress (Butterfield and Boyd-Kimball, 2005). Further models in *C. elegans* also suggest that the pre-fibrillar Aβ species are responsible for the oxidative stress, not the Aβ fibrils themselves, which are composed of parallel-stacked Aβ peptides that form a cross-β-sheet structure (Drake et al. 2003). However, the “senile plaques,” as described by Alois Alzheimer, are significant in size and physically impede neurological functioning (Alzheimer, 1907). Because of this, the mechanism behind why fibrils form in the first place is crucial for understanding this hallmark of the Alzheimer’s disease pathology, especially since much debate has taken place as to which form of Aβ is the most detrimental to a healthy brain, whether it be the pre-fibrillar species, oligomeric intermediates, or the plaques themselves.

Within the fibrils, as well as oligomers, metal ions such as iron, zinc, and copper have been found in significant concentrations, and though more experiments must be done to discern why this is the case, this may be tied to ROS activity since Cu²⁺ has the ability to catalyze the production of ROS and radicals (Butterfield and Boyd-Kimball, 2005; Kowalik-Jankowska et al., 2002). The binding of copper to Aβ 1-40 has been shown to have a 1:1 stoichiometric ratio with the peptide with a binding constant at physiological conditions (37 °C, pH 7.4) of 2.4 ± 0.2 × 10⁹ M⁻¹ (Hatcher et al., 2008). If the binding of Cu²⁺ induces or aids the reduction-oxidation reactions resulting in neurotoxicity, which is suggested by evidence in this case, then analyzing the reactions of metals binding to Aβ may be of potential therapeutic use in the form of chelation agents (da Silva and Ming, 2007; Guilloreau et al., 2007; Smith et al., 2006). Of additional interest is the role of metallic ions in the plaque formation itself, if the metal-Aβ peptide complex results in a variance in the structure or rate of plaque formation. Work by Ha et al. focused on this phenomenon.
as applied to deposition onto a solid surface, and this should be expanded into aqueous suspensions of Aβ, preferably with a variance of time for introduction of the metallic ions to determine the metals’ effects over the course of fibril formation (Ha et al., 2007).

**Fibril Formation**

Aβ is known to form in a fibrillar manner that is a primary ingredient in the composition the interneuronal plaques that form along the pathological course of Alzheimer’s disease (Glenner and Wong, 1984). There have been multiple proposed mechanisms of Aβ fibril formation from its monomeric form, but overall, the exact mechanism of assembly is largely unknown, though dimers, oligomers, and protofibrils have been observed as intermediates (Kelly, 2000). Nucleation models have been proposed, where peptides are added onto an existing “nucleus,” or small fibril, though the rates of formation are extremely dependent on shape and size of the nuclei (Friedrich et al., 2010). At physiological conditions, the Aβ monomers at micromolar concentrations have a disordered structure, where a coil to β-sheet structural transition can occur; a coil to α-helix conformational change has also been proposed for fibrillization, though this assertion has been under scrutiny (Nalivaeva and Turner, 2013; Fezoui and Teplow, 2002). Oligomeric forms of Aβ are presumed to be largely responsible for fibril and protofibril formation (Harper et al., 1999).

An important observation consistent among experimental data is the existence of two distinct phases, a lag phase and a growth phase (Figure 2). While there are individual variations observed between the individual rates of fibrillar quantitation, there is always a lag time before fibrillar expansion, signifying that growth is not linearly dependent on the original concentration of a monomer in solution but that there must be some intermediate step or process occurring before fibrillar growth becomes significant, at least according to the measures of quantization used (Friedrich et al., 2010). This is consistent with the assertion that the stacking of the oligomeric form of Aβ is responsible for the increase in growth, where dimers and oligomers are formed in the lag phase and oligomers in the growth phase combine to form fibrils.

![Figure 2: Fluorescence-monitored Aβ 1-40 fragment aggregation over time.](image_url)

What must also be practically considered is that the measurement of Aβ fibrillar formation requires a method of fibril quantification. Accurate fibril quantification is a necessity in any experiment that has the aim of determining whether or not a therapeutic agent is effective in breaking down Aβ fibrillar structures. Depending on the exact nature of desired analysis, there are a variety of potential methods available for visualization and/or analytical quantitation, such as atomic force microscopy, fluorescent labeling of the peptide, staining methods, and the use of fluorescent probes that increase in intensity upon interaction with Aβ fibrils.

**Atomic Force Microscopy**

Since Aβ fibrils have a distinct shape, as compared to the smaller oligomers, atomic force microscopy (AFM) has been utilized for monitoring the rate of Aβ fibril assembly by way of a topological map images over time (Jungbauer et al., 2009). Traditionally, AFM has been used for peptide species whose formations are morphologically distinct, which makes this technique useful for monitorization of Aβ fibril formation (Ha et al., 2007; Chaney et al., 2005). Images produced are quite detailed due to the high resolution of AFM that can get as low as 0.6-1.0 μm, but because of this only a few fibrils may be analyzed within the frame (Park, 2020). Therefore, AFM may be useful in Aβ research circumstances monitoring Aβ fibrilization in tissue samples.
**Direct Fluorescent Labeling**

Aβ may be labeled on the N-terminus end by attachment of a fluorophore, such as fluorescein or its derivatives, HiLyteFluor™488, AlexaFluor®488, tetramethylrhodamine, or Cy3/Cy5 and their derivatives, for Aβ fibril quantification (Jungbauer et al., 2009). This labeling may occur before or after fibrilization is initialized. However, there are some considerations that must be taken into account for each route of Aβ labeling. If the peptide is previously labeled, the final form of the fibril is altered in structure, but labeling the peptide post-fibrilization results in extraneous dye present and an unknown stoichiometric ratio between labeled and unlabeled peptides (Jungbauer et al., 2009). This method is therefore not particularly of analytical utility in many cases of tissue-derived samples and should still be done with care when used for *in vitro* applications. That said, fluorescent labeling after oligomerization occurs may be useful for quantitation since labeling at this point would not affect the oligomeric formation process.

**Congo Red**

Congo Red is a histological dye that, upon binding to the β-sheet structure of peptide fibrils, experiences an alteration in the wavelength of maximum emission (Klunk et al., 1999). From this shift, the amount of Congo Red that is bound to the fibrils may be analyzed, thus quantifying the amount of Aβ fibrils that are present in the sample being studied. Additionally, Congo Red may be used for quantification of Aβ plaques present in postmortem brain tissue samples, as binding of the stain occurs on the Aβ fibrils as opposed to loosely-associated peptides (Wilcock et al., 2006). The association between Congo Red and Aβ fibrils does not just apply to postmortem tissue; the dye also has *in vivo* imaging applications (Frid et al., 2007). While the original purpose of Congo Red was meant for use as a stain, or alternatively for spectrophotometric quantification of Aβ fibrils, the dye has also been implicated as an inhibitor of fibril formation (Lorenzo and Yankner, 1994). This point does call into question Congo Red’s use for quantifying Aβ fibrilization: if Congo Red itself destabilizes Aβ fibrils, then the kinetics of this destabilization process must be researched to obtain a procedural time frame in which the fibril quantification maintains analytical accuracy.

**Thioflavin T Fluorescence Assay**

Fluorescence itself occurs when electromagnetic radiation is absorbed by a fluorophore, or compound that fluoresces, and then a photon is subsequently and spontaneously emitted, usually at a slightly longer wavelength than the wavelength at which the absorption occurred in this context. As the fluorescence signal intensity is directly proportional to the amount of fluorescence occurring, the signal intensity may be related to the number of circumstances that allows for fluorescence to occur. Originally developed in 1965, the Thioflavin T (ThT) fluorescence assay has been a prevalent tool in the quantification of Aβ fibrils, as ThT becomes significantly more fluorogenic upon binding to the cross-β-sheet structure of the Aβ fibrils (Rogers, 1965). Portions of the molecular structure for ThT (Figure 3) are aromatic in nature, and the difference in energy between the highest occupied molecular orbital and lowest occupied molecular orbital due to the π-bonding system corresponds to a wavelength of maximum absorption of 412 nm in aqueous solutions (Maskevich et al., 2007).

Upon binding to the Aβ fibrils, the wavelength of maximum absorption increases to 450 nm, and the wavelength of maximum emission shifts to approximately 480 nm (Maskevich et al., 2007). Because of the shift in maximum absorption, the result is a visible increase in intensity at 480 nm as ThT binds to the Aβ fibrils (Figure 4). The fluorescence signal intensity shift thus proportionally corresponds to the binding of ThT to the Aβ fibrils. This increase in intensity is due to the emission of a photon at 480 nm when ThT binds to the Aβ fibrils, resulting in a visible increase in fluorescence intensity at 480 nm.
fibrils. This may be monitored over time to determine the relative rate of fibril formation; the greater amount of signal intensity, the greater number of opportunities for ThT to bind to Aβ fibrils, assuming that equilibration time and ThT concentration are kept constant. Addition of excess ThT will result in only a slight increase in the emission signal, as at that point the excess physically cannot be bound to the fibrils and thus remains unbound.

Figure 4: Fluorescence emission spectra for solutions of unbound ThT, Aβ peptide 1-40, and ThT with Aβ 1-40.

Since the magnitude of increase between the fluorescence of unbound ThT and ThT bound to Aβ fibrils is dramatic, almost twelve-fold in a conservative example (Figure 4), the presence of Aβ fibril binding opportunities for ThT is easily identifiable and quantifiable.

**Assay Considerations**

While the ThT assay for detection and measurement of Aβ fibrils is a relatively simple procedure, there is an important principle that must be maintained: any other reagent utilized in solution must not affect the magnitude or wavelength of maximum emission. For this primary reason, the ThT fluorescence assay has come under fire when working with the testing of aromatic compounds as potential fibril destabilizers, as bias in the fluorescence intensity may lead to incorrect conclusions on the Aβ fibril destabilization efficacy of the target compound under study. This has been shown to be the case for some polyphenols, such as quercetin, resveratrol, and curcumin, where the fluorescence output is altered in the presence of ThT (Hudson et al., 2010). However, there are multiple means by which interference can occur. If there is unwanted interaction between ThT and the compound under study, or the compound is directly blocking the binding sites for ThT and that fluorescence signal output is not a representative result of fibril destabilization, such as is the case with Rifampicin, then the assay cannot be used for an accurate measure of fibril quantification (Meng et al., 2008). However, if the signal intensity shift is simply due to an increase or decrease from the addition of the desired compound and the binding process of ThT is not hindered, the assay may still be used for accurate quantification of Aβ fibrils formed as long as the variable of the compound addition is taken into consideration as necessary. Here is where the primary point of controversy lies, for the ThT assay must theoretically remain sound upon the addition of the molecule under study in order to maintain Aβ quantification accuracy. When verified to give reliable Aβ quantification, the amount of Aβ fibrilization may be tracked over time studies to obtain further information about the kinetics and mechanisms of Aβ fibril formation. With this data, the manner in how plaque formation occurs in the context of Alzheimer’s disease may be further elucidated.

**Polyphenols**

Polyphenols are a class of molecular compounds that happen to be known antioxidants. Several molecules of this category have been shown to be useful in the treatment of cardiovascular disease, cataracts, and neurodegenerative diseases (Morimitsu et al., 2002). For this reason, many polyphenolic compounds have been and are currently being investigated as potential therapeutic agents that could be utilized for the treatment of Alzheimer’s disease. Here, the sourcing of polyphenols, their structural diversity, and their use in Aβ fibrilization studies shall be discussed.

**Sourcing**

There are over 8000 known polyphenols, many of which are commonly found in plants and plant-based materials that can be readily consumed, such as vegetables, fruits, grains, and coffee beans (Harborne, 2013; Dragovicuzelac et al., 2007). An added benefit
of polyphenols for this type of treatment is their abundance in foods and supplements since polyphenols are naturally-occurring compounds (Archivio et al., 2007; Scalbert et al., 2005). The average consumption of polyphenols per day is about 1 g; however, the bioavailability is often much less, as the large structure of many polyphenols discourages absorption into the bloodstream by way of the small intestine. After consumption of 10-100 mg of an individual polyphenol, the resulting blood plasma concentration of the polyphenol is about 1 μM (Kühnau, 1976; Scalbert and Williamson, 2000). Therefore, working concentrations for analysis should be based on concentrations within the general realm of 1 μM, or slightly higher as would be applicable to a specific route of treatment that would be administered in addition to the average diet consumption.

**Polyphenol Structure**

As the name implies, polyphenols are compounds containing multiple phenol groups, and the sub-classification of the polyphenol class can get quite complex (Figure 5). There are several major categories, including phenolic acids, flavonoids, stilbenes, phenolic alcohols, and lignans (Tsao, 2010). A great amount of variety is present within the general classification of polyphenol; because of this, there are many differences in chemical behavior that must be investigated independently for each compound. Of particular interest in research are the categories of flavonoids and stilbenes, as many of these compounds are known to have antioxidant properties, which mitigate and minimize the amount of potential damage that can be done by reactive oxygen species (Rivièrè et al., 2008; Ono et al., 2002).

![Figure 5: General classification of polyphenols.](image-url)

The largest grouping of polyphenols is the flavonoids, containing over 4000 of the known polyphenols (Harborne, 2013). Flavonoids are characterized by two aromatic rings, each containing at least one hydroxyl group, as well as a heterocyclic system containing a three-carbon chain between the aromatic rings, and there is a great variety of structures within the flavonoid family (Figure 5; Figure 6). Some flavonoid compounds under investigation include quercetin and (-)-epicatechin. Quercetin is plentiful in onions and has been shown to inhibit amyloid structural formation and cross the blood brain barrier but not affect gene transcription relevant to Alzheimer’s disease, whereas (-)-epicatechin is a polyphenol in cocoa found to inhibit apoptosis induced by Aβ (Wang et al., 2011; Huebbe et al., 2010; Heo and Lee, 2005). However, more research must be done to draw conclusions on usefulness as a therapeutic measure, as well as to investigate potential side effects, since most experiments are done at concentrations around 25 μM, substantially greater than the human blood plasma concentration after average meal ingestion. For (-)-epicatechin, effects of hepatotoxicity have been tangentially noted for green tea extract supplements containing (-)-epicatechin, though the supplements also
contain additional polyphenols such as epigallocatechin and are significantly more concentrated than the typical 15 mg (-)-epicatechin in an average glass of green tea (Dasgupta, 2019).

<table>
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<tr>
<th>Anthocyanidins</th>
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<tr>
<td>Cyanidin</td>
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<td>Naringenin</td>
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<td>Flavonols</td>
<td>Isoflavones</td>
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<td>Quercetin</td>
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**Figure 6:** Example structures of various flavonoid classifications.

Stilbenes (Figure 7) are characterized by a general structure of two phenolic groups capping the ends of an inner carbon chain, with hydroxyl placement and substituents providing variation within the category (Scalbert et al., 2005). This classification group includes molecules such as resveratrol, which is found in high concentrations in grape skin and has been shown to reduce the amount of Aβ fibrilization over 72 hours at a concentration of 40 μM (Marambaud et al., 2005). However, further investigation should be conducted at lower concentrations of resveratrol so that polyphenol species can be compared on similar grounds with regard to concentration, as most studies are conducted within the range of 10-30 μM (Wang et al., 2011; Guzmán-Beltrán et al., 2013).

**Figure 7:** Molecular structure of resveratol.

While many polyphenols are currently being studied, the structural characteristics of relative planarity, two aromatic end groups, and hydroxyls that can be involved in hydrogen bonding, make stilbenes ideal candidates for Aβ disaggregation. For this reason, stilbenes are of particular interest to many for future experimentation (Ono et al., 2002; Reinke and Gestwicki, 2007).

**Mechanisms of Interaction with Aβ**

As far as interactions with Aβ fibrils are concerned, there must be mechanisms by which fibril stabilization and destabilization may occur. Within the fibril itself, aromatic residues aid in stabilization of the trademark cross β-sheet structure (Reinke and Gestwicki, 2007). There has been postulation that antioxidant activity of the polyphenols is the primary mechanism, but structure does seem to also play a significant role in efficacy, as oxidative stress from hydrogen peroxide was not truly impeded by the presence of polyphenols (Conte et al., 2003). Molecular dynamics simulations have found that the polyphenols interrupt the H-bonding in the oligomeric form of the Aβ aggregate, though this was specifically pertaining to curcumin, exifone, and myricetin and needs to be expanded to a greater variety of structures to investigate all potential mechanisms of interaction (Berhanu and Masunov, 2015). Investigation into the structural characteristics of ligands implicated in Aβ disassembly found that any modification to the presence of two aromatic end groups, any substituents, and length of the linking region between the aromatic groups drastically affected inhibition rates, with an optimal linking chain length from 6-19 Å (Reinke and Gestwicki, 2007). This information may be utilized for the development of molecular options for molecular dynamics simulations in order to determine what
molecules may best disaggregate the Aβ fibrils, with optimal compounds inspiring the direction of future experiments. From this, polyphenolic therapeutic options for the disaggregation of Aβ fibrils to break down or slow down the formation of the interneuronal plaques of Alzheimer’s disease may be identified or developed.

**Challenges of Analysis**

As previously mentioned, if a ThT fluorescence assay is utilized for Aβ quantification, there must be no factors inhibiting the binding between ThT and the fibril, nor should there be any interaction between ThT and the introduced compound that increases or quenches the fluorescence signal intensity. However, this has become a point of controversy, for some polyphenols have significant absorbance within the visible light spectrum and can fluoresce significantly depending on the wavelength of excitation. Because of this, the emission spectra of polyphenolic compounds under scrutiny should be obtained with and without the presence of Aβ, ThT, or both in order to assess viability of the ThT fluorescence assay to produce an accurate representation of Aβ aggregation. The use of computational methods to determine the relative binding strength to Aβ fibrils of ThT and any polyphenol compounds studied would be beneficial for ascertaining the presence of any significant molecular interference that may affect the analytical integrity of the ThT assay. If the ThT assay is determined to be inaccurate in the presence of a certain polyphenol, other methods should be utilized for Aβ fibril quantification.

**Curcumin: A Closer Look**

**History and Background**

One polyphenolic compound that has recently been emphasized in studies as a potential treatment for Alzheimer’s disease is curcumin, specifically for use as an attenuation agent of Aβ fibrilization. Curcumin is a stilbene that is symmetric in structure (Figure 8), derived from the *Curcuma longa* plant, the root of which is commonly known as turmeric, a spice that is ubiquitous in Indian and Southeast Asian cooking (Aggarwal et al. 2005).

![Figure 8: Structure of curcumin.](image)

The toxicity of curcumin is relatively low, with a curcuminoid-essential oil complex having no adverse symptoms or mortality at the maximum dose level of 5000 mg kg⁻¹ in rats and mice, which is substantially higher than that which would be present within a normal diet (Aggarwal et al. 2016). Also, in a clinical setting, curcumin was administered in a bioavailable form at 90 mg/12 hr over the course of 18 months in a double-blind trial, with a result of improvements in memory and attention and decrease in plaque accumulation (Small et al., 2018). Curcumin has additionally been the subject of human toxicity studies in which a dose of 8000 mg was given every day over the course of three months with no adverse symptoms reported (Cheng et al., 2001). As curcumin is already known to be safe for human consumption over an extended period of time and has served to treat the sharpening of memory, the progressive loss of which is a symptom of Alzheimer’s disease, it is of interest as a potential therapeutic measure given these practical perspectives.

Curcumin has been traditionally used as medicine for anti-inflammatory, antitumoral, and antimicrobial purposes (Noureddin et al., 2019). Due to these properties, investigation was inspired for curcumin’s potential use as an Aβ fibril destabilizer. Current research has found that a low dose of curcumin in a rat’s diet stimulates microglial search and capture by phagocytosis of Aβ plaques, aiding in plaque clearance, although the higher dosage studied did not display this effect (Teter et al., 2019). Additionally, due to the ability to bind to Aβ fibrils, curcumin has been conjugated to magnetic nanoparticles for use in MRI imaging, in order to visualize Aβ plaques for diagnostic purposes (Cheng et al., 2015).
In a laboratory setting, curcumin has also been utilized as a probe for detection of other protein aggregates, such as bovine serum albumin, by taking advantage of the alteration in curcumin’s emission profile upon binding to Aβ (Karmakar et al., 2019). Since curcumin has also been utilized as an Aβ fibril labeling dye in vivo and in postmortem tissue, this qualitative aspect must be taken into consideration for any experiments dependent upon spectroscopic properties that are relevant to the wavelength range being utilized (Maiti et al., 2016).

Specific Challenges of Analysis

Curcumin has a wavelength of maximum absorption at approximately 424-425 nm at 298 K in aqueous solutions (Chignell et al., 1994). The temperature must be taken into consideration, as the absorbance spectrum changes drastically with variance in temperature (Liu et al., 2012). As this results in a fluorescence spectrum with considerable overlap to the peak produced by ThT, many doubt the efficacy of the ThT assay for monitoring the effect of curcumin on Aβ fibril aggregation rate; to compensate, Nile Red has been proposed as an alternative fluorescent dye for determination by anisotropy (Nedaei et al., 2018). Current investigations in our lab have determined there to not be a significant impact of fluorescence quenching in the wavelength range monitored for the Thioflavin T range of maximum emission, though there does seem to be significant quenching of the curcumin peak and a hypsochromic shift in the wavelength of maximum emission (Figure 9). This is most pronounced as the addition of ThT increases beyond the amount utilized in the procedure for our lab. However, the increase in fluorescence signal does still directly correlate with the addition of ThT in the region of interest, 476-484 nm. Therefore, the addition of ThT will still result in an increase in fluorescence intensity regardless of the presence of curcumin, especially for the ThT addition experimentally utilized, and thus the ThT fluorescence assay does theoretically hold in this regard. Further analysis must be conducted to determine binding affinities between ThT, curcumin, and Aβ in order to verify that curcumin is not blocking the direct binding sites for ThT on the fibrils during the assay, as then the concentration of Aβ fibrils will be indirectly underestimated and thus compromise the integrity of the assay results.

As previously mentioned, the emission spectrum of curcumin is altered upon binding to Aβ fibrils (Karmakar et al., 2019). Therefore, with knowledge of the overall absorbance at various wavelengths for curcumin, curcumin with Aβ, and curcumin with Aβ and ThT, absorbance may theoretically be monitored at multiple wavelengths over equilibration time to determine if ThT or curcumin displays a greater binding preference in the case of binding site competition. This information would provide insight as to the nature of Aβ interaction strength with ThT and curcumin and may be used for determining viability of the ThT assay accuracy in the presence of curcumin.

Conclusions

Ultimately, the goal of this field of research is to find a treatment to prevent or slow down the progression of Alzheimer’s disease. The Aβ fibrillation process, a hallmark of the disease’s pathogenesis, has been extensively studied. However, there is still information that is not known about the fibril assembly process and its exact role. Past research suggests that oligomers are formed before the oligomers themselves assemble into the large fibrillar structure that builds up the majority of the plaques, and studies previously mentioned have noticed a trend of fibril formation increasing substantially after an initial period in which...
oligomers are primarily being formed (Harper et al., 1999; Friedrich et al., 2010). Quantitation of these Aβ fibrils and plaques can occur by a variety of means, including methods of microscopy, fluorescence spectroscopy, and many more. The ideal method for utilization depends on the sample type and any interfering species present, such as polyphenols, and the chemical nature of any additional compound abiding in solution with Aβ must therefore be considered when determining the method of Aβ fibril quantitation.

Overall, the current body of work supports postulations surrounding the use of polyphenols, especially curcumin, as potential therapies for Alzheimer’s disease by preventing and/or destabilizing the Aβ fibrils that compose the majority of the interneuronal plaques implicated in the pathology of the disease. However, there is an immense variety of structures within the polyphenol family; focusing on stilbene compounds may be useful as a starting point (Tsao, 2010). Concerning the analysis of spectroscopic data, every compound has slightly different properties with regards to the absorption and emission of photons, and this must be taken into consideration for decisions on how quantification of Aβ fibrils should be experimentally collected. This pertains especially to the ThT fluorescence assay protocol, and there must not be any intermolecular interactions that bias the fluorescence signal intensity, such as undue quenching or interference due to shifting in the emission profile of a polyphenol upon binding (Berhanu and Masunov, 2015).

Future work with the goal of elucidating a therapeutic method that attenuates the progression of interneuronal plaque formation should focus on the effect of curcumin on the Aβ aggregation rate for fibrils composed of different segments of the Aβ peptide in order to determine the location of curcumin’s interactions with Aβ to aid in the ascertainment of the mechanism(s) of interaction and utility as a therapeutic agent. While full-length Aβ segments are the peptides biologically present in vivo, information on the aggregation of Aβ fragments taken from both the extracellular and intermembrane portions shall provide insight on the section of Aβ that curcumin interacts with, specifically the nature of the residues with regards to polarity. Thus, this is a potential avenue of investigation to better solidify which portion of the overall Aβ full-length peptide is primarily impeded by the presence of curcumin—the formation of oligomers or the subsequent formation of fibrils (Mills and Reiner, 1999). An additional computational investigation should also be conducted into the accuracy of the ThT assay in the presence of curcumin by comparison of binding affinities of ThT and curcumin to Aβ fibrils. With the aforementioned factors taken into consideration, a more solid empirical foundation may be established for the utility of curcumin, or another polyphenolic therapeutic candidate, as a treatment for Alzheimer’s disease.

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Corresponding Author

Brittany Hagenhoff
Truman State University
100 E. Normal St
Kirksville, MO 63501
BLH50730@gmail.com

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