Synthesis And Characterization Of Acetaminophen-Derived Nanoparticles: A Novel Approach To Inhibit Fibril Formation

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SYNTHESIS AND CHARACTERIZATION OF ACETAMINOPHEN-DERIVED NANOPARTICLES: A NOVEL APPROACH TO INHIBIT FIBRIL FORMATION

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Dean of the Graduate School
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Hannia Elena Mendoza-Dickey
Dedication

I dedicate this thesis is to my loving family and my husband. Your unwavering support, encouragement and infinite love have been my pillars of strength. Without it, my dream would not have been possible. I will always be eternally grateful.
SYNTHESIS AND CHARACTERIZATION OF ACETAMINOPHEN-DERIVED NANOPARTICLES: A NOVEL APPROACH TO INHIBIT FIBRIL FORMATION

by

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THESIS

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Abstract

In the realm of nanotechnology, nanoparticles (NPs), have garnered significant notoriety in recent scientific research due to their unique physical and chemical properties, such as fluorescence emissions, nanoscale dimensions (typically <1000 nm), ease of surface modification, and biocompatibility. Nanoparticles have shown their potential across a variety of areas, including advanced industrial applications and cutting-edge biomedical research. Considering their cost-effective synthesis, they have shown promise as therapeutic agents for a variety of bioimaging and biomedical applications. This thesis describes the synthesis and detailed analysis of acetaminophen-derived nanoparticles. Techniques such as Dynamic Light Scattering (DLS), Thioflavin T (THT) assay, Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR), $^1$H NMR spectroscopy, and Ultraviolet-Visible Spectroscopy (UV-VIS) were utilized for structural and functional assessments. Acetaminophen derived nanoparticles (ANPs) exhibit potential to hinder the amyloidogenic conversion of soluble amyloid-forming proteins into their toxic form. The novelty of this research focuses on the utilization of chemical structures capable of traversing the Blood Brain Barrier (BBB) to mitigate xenotoxicant-induced neuronal damage, a notable contributor to neurodegenerative disorders. This thesis describes the synthesis and characterization of acetaminophen derived-nanoparticles (ANPs). Our nanoparticles possess anti-amyloidogenic properties as evidenced by their ability to disrupt in the soluble-to-toxic trajectory of HEWL.
The prevalence and evolution of amyloid fibrils are consistent features in the pathology of neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer’s Disease (AD), and Huntington’s Disease (HD), as well as metabolic disorders like Type 2 diabetes (T2D). The relationship between amyloidogenic pathways and these disorders highlights the imperative for enhanced understanding and the formulation of specific therapeutic interventions.
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Chapter 1: Introduction

OVERVIEW OF NEURODEGENERATIVE DISEASES

Aging is the primary risk factor for the onset of neurodegenerative diseases, such as AD, PD, HD, frontotemporal lobar dementia (FTD) and systemic lysozyme amyloidosis.\textsuperscript{1,2} The term “Neurodegeneration” is derived from two words: the prefix “neuro-,” refers to nerve cells (i.e., neurons), and “degeneration,” denoting the progressive decline of structures, and/or functionality in tissues or organs.\textsuperscript{3} This refers to the ongoing dysfunction and deterioration of neural synapses, accumulation of physiochemically altered protein variants in the brain, and the subsequent loss of neurons and their function within areas of the central nervous system (CNS).\textsuperscript{4} Consequently, this leads to either physical disability, cognitive decline, or both. Around the world, people are living longer—well into their 80s, on average—and the occurrence of neurodegenerative diseases tends to rise significantly within geriatrics.\textsuperscript{5,6} For instance projections for Alzheimer’s disease, which is the most common among these conditions and currently affects approximately 15 million people globally, suggest that the number of affected individuals in the United States could rise to 13.2 million and in Europe to 16.2 million by 2050.\textsuperscript{7,8}

Many pharmaceuticals have been approved for neurodegenerative diseases, but their long-term use has been shown to cause debilitating side effects, and none appear to prevent degenerative diseases from occurring. With this in mind, it is imperative to explore and develop novel therapeutic strategies in particular those that possess unique physicochemical properties that make them promising targets for the treatment of neurodegenerative diseases.
Amyloidogenic Protein Misfolding

Misfolded amyloidogenic proteins such as amyloid beta (Aβ), α-synuclein, mutant Huntington Protein (mHTT) and islet amyloid polypeptide (IAPP) among others have the propensity to transition from their soluble and monomeric states to toxic aggregated forms. These aberrant proteins are typically disease specific as seen in disorders such as AD, PD, HD and T2D, respectively.2,9 This aggregation typically represents the culmination of the amyloidogenic pathway. During this process, the polypeptide chains undergo structural modifications through secondary processes that lead to the maturation and assembly of amyloid fibrils, which are rich in beta (β)-sheet structure in their polypeptide backbones.10,11 This irreversible structural arrangement endows them with resistance to proteolytic degradation and renders them insoluble.12 As aforementioned, these aberrant proteins are typically disease specific. For example, Aβ and Tau proteins are implicated in AD. Accumulation of these proteins in the brain can lead to plaques and neurofibrillary tangles (NFTs), which impair synaptic communication “neuron-to-neuron communication” and contribute to the degradation of cognitive function and neuronal death.13
Illustration 1: Senile plaques are located between neurons in the brain. The accumulation of these plaques is one of the hallmark characteristics of Alzheimer's disease, along with neurofibrillary tangles. While predominantly composed of Aβ protein, these plaques may also be composed of other components.

In PD, Lewy bodies are a characteristic component. A principal component of these Lewy bodies includes α-synuclein. These molecules can form small, repeated units called oligomers or longer fibrils. These fibrils are toxic to neurons and play a key role in driving PD. Visible features in PD are muscle rigidity, tremors at rest, and slowness of movement.\textsuperscript{14}

mHTT represents a modified variant of the native Huntington protein, critically implicated in the pathophysiology of HD. The mutation in the HTT gene causes a propensity for the protein molecules to aggregate. This formation of mHTT fibrils has characteristics that are like that of amyloids, where the polyQ segment of mHTT establishes a densely structured, rigid, and dehydrated anti-parallel sheet core surrounded by the N- and C-terminal domains.\textsuperscript{15} These aggregates subsequently contribute to neurotoxicity, hastening the demise of neurons. This neuronal loss is pivotal in the manifestation of the characteristic motor and cognitive dysfunctions observed in individuals affected with HD.\textsuperscript{16}
T2D is a chronic metabolic disorder linked to the accumulation of misfolded protein aggregates. T2D is associated with obesity via increased insulin resistance. While not directly classified as a ND, evidence suggests it contributes to the development and increased risk of protein misfolding disorders (PMDs), including diseases that affect the CNS namely, Alzheimer’s and Parkinson’s diseases. The exact mechanism of protein misfolding and associated toxicity remains unclear.  

The IAPP, commonly known as amylin, is a peptide hormone that is co-secreted with insulin by pancreatic β-cells. In glycemic homeostasis, it acts by slowing down the gastric motility, which increases the sensation of satiety by enhancing the sensation of fullness. This action contributes to the mitigation of postprandial elevations in blood glucose levels. In T2D, IAPP can aggregate to form amyloid deposits within the pancreatic islets. These islet amyloid deposits contribute to β-cell dysfunction and apoptosis, exacerbating TD’s poor insulin secretion. The accumulation of amylin in the circulatory system, which constricts blood vessels and potentially compromises cortical blood flow. There is evidence suggesting that vascular cell dysfunction might be associated to the progression of AD pathologies, specifically the progression of both amyloid-beta (Aβ) and tau protein aggregations. Should amylin be confirmed as a contributor to AD pathology, strategies focusing on preventing its aggregation, or its passage across the blood brain barrier (BBB) could emerge as promising therapeutic avenues.
**Current Challenges Treating the Blood Brain Barrier**

The blood brain barrier (BBB) is a complex and tightly regulated endothelial membrane, fortified by pericytes and other elements that is important in maintaining the CNS. Predominantly constituted by cerebral microvasculature endothelial cells, the BBB is pivotal for CNS homeostasis. Its structural and functional integrity is further supported by specialized cells such as pericytes, astrocytes, and neighboring neurons. These cellular components communicate through complex junctions: tight junctions (TJs), adherent junctions (AJs), and gap junctions (GJs). Notably, TJs play a crucial role in establishing and augmenting the trans-endothelial resistance intrinsic to the BBB. Collectively, these junctions reflect the integrity and permeability of the barrier as they bind tightly to adjacent cells, thereby occluding the intercellular space between them. These junctions collectively confer structural and functional defense against any pathogens that may be circulating the bloodstream. By doing so, it maintains a controlled environment essential for neuronal function and interaction, overseeing the precise entry and exit of molecules to preserve brain homeostasis. It is challenging for pharmaceutical drugs to penetrate the CNS due to the physiological function and stringent regulation of the BBB, which is the primary reason for the complications encountered in existing treatment strategies and the numerous research studies geared towards the development of novel drug delivery systems for targeting and treating brain diseases, such as NDs and cancer.
THESIS CONTRIBUTION

This thesis covers the following research objectives: (i) The synthesis of nanoparticles via hydrothermal processes utilizing Acetaminophen as a precursor. (ii) The elucidation of inhibitory properties of ANPs against amyloid fibril formation, assessed through Thioflavin T (ThT) fluorescence assays. (iii) The assessment of the structural integrity of the precursor material subjected to hydrothermal synthesis under varying durations of 4, 6, 8 hours, emphasizing the influence of heat. (iv) A detailed analysis of the resultant nanoparticles employing techniques such as Dynamic Light Scattering (DLS), UV-Visible Spectroscopy (UV-VIS), ATR Infrared Spectroscopy (ATR-IR), Proton Nuclear Magnetic Resonance (\(^1\)H NMR).

Limitations of polyphenolic compounds

To date, pharmaceutical interventions mainly aim to alleviate the symptoms of neurodegenerative diseases and fail at targeting their underlying pathogenesis and offering a curative approach. A pressing need exists to explore innovative therapeutic methods that can effectively treat these debilitating conditions. Particularly, polyphenols have been recognized for their potential therapeutic applications in various chronic diseases as well as their strong antioxidant properties, which enable them to quench free radicals and counteract oxidative stress.\(^{24}\) These compounds represent naturally occurring bioactive molecules prevalent in a wide variety of foods and botanicals.
They are also effective in reducing neuroinflammation, protecting neurons, and enhancing cognitive functions. Flavonoids represent a prominent class within the four principal classes of polyphenols, alongside phenolic acids, lignans, and stilbenes. These organic molecules are characterized by the presence of at least one aromatic ring that is connected to one or multiple hydroxyl functional groups, manifesting as several hydroxyl constituents attached to the aromatic ring. Unfortunately, many aromatic polyphenols have limited bioavailability, meaning they are inadequately absorbed, digested, and dispersed throughout the body. Even though many aromatic polyphenols may exhibit beneficial and potent effects in vitro, there is no guarantee that similar therapeutic benefits will also be observed in vivo. This is because the compound does not reach the target tissues in sufficient concentrations to provide a therapeutic effect. As previously mentioned, the blood-brain barrier (BBB) serves as a selective barrier to protect the brain, and numerous substances, including aromatic polyphenols, encounter challenges in traversing it.

Given their proclivity for non-specific binding, aromatic polyphenols can interact with a broad spectrum of biological receptors. This can potentially result in unforeseen reactions, which might compromise the intended therapeutic outcome. In conclusion, researchers continue to deepen their knowledge of these compounds and how they are absorbed by the body, aiming to craft successful treatments. There remains potential for certain aromatic polyphenols, or their derivatives, especially when combined with other therapeutic strategies or introduced using methods that address the previously mentioned obstacles.
Biomedical Applications of Carbon-Based Nanomaterials

Carbon, a fundamental element, is ubiquitously present on Earth and is crucial for sustaining life. At the molecular level, carbon’s ability to form covalent bonds manifests in diverse configurations, dictating its distinct material properties. In the realm of biotechnology, carbons prowess is redefined. Carbon nanomaterials (CNMs), including carbon quantum dots, nanodiamonds, carbon nanotubes, carbon nano onions, and various graphene-based derivatives possess a myriad of structural properties. These unique characteristics endow them with exceptional mechanical, electrical, thermal, optical, and chemical attributes.²⁹,³⁰

There have been numerous applications of these materials, particularly in the field of biomedicine, as exemplified by the application of carbon nanoparticles (CNPs), which represent the cutting edge of biomedical fields. A major advancement in this research is precisely delivering drugs embedded into their membranes to target specific brain sites — imperative for treating neurological disorders. CNPs has been also used in biomedical imaging, biosensors, tissue regeneration and cancer therapy.³⁰ There is no doubt that these nanoparticles are very small, measuring between 1 and 1000 nm in at least one dimension; however, they are commonly defined as having a diameter ranging from 1 to 100nm.³¹ Putting them in the same size regime as biological macromolecules like proteins and viruses. As the pharmaceutical sector contends with the translational gap between in vitro findings and in vivo efficacy, CNPs emerge as a promising and cost-effective solution, potentially bridging this chasm and optimizing drug delivery to target desired disease sites.
Investigating Acetaminophen as a Therapeutic Strategy

Acetaminophen, (APAP - also known as paracetamol in many countries) is widely recognized for its efficacy in alleviating fever and discomfort. It remains a predominant choice for over-the-counter medicinal interventions both in the United States and Europe. Typically employed to counteract mild to moderate pain symptoms, this therapeutic agent falls under the category of non-acidic antipyretic analgesics. Furthermore, in scenarios for easing of severe pain—like post-operative pain or pain resulting from cancer—it is often administered in conjunction with opioid analgesics to enhance its pain-relieving potential.

It is preferred to prescribe acetaminophen when non-steroidal anti-inflammatory drugs (NSAIDs) are deemed unsuitable for patients. As NSAIDs tend to cause gastrointestinal irritation, acetaminophen is generally better tolerated by individuals with gastric ulcers or bronchial asthma. Moreover, acetaminophen is a safer choice for analgesics, and fever reduction in pregnant women and nursing mothers, since it is considered relatively less likely to cause adverse effects during pregnancy and breastfeeding. It is also one of the most well-established analgesics for managing pain in children.

The mechanisms by which acetaminophen exerts its pain-relieving effects remain subjects of ongoing research. Initially, it was posited that acetaminophen inhibited the enzyme cyclooxygenase (COX) causing its analgesic effects. According to contemporary understanding, acetaminophen undergoes metabolic transformation into p-aminophenol, a substance capable of crossing the blood brain barrier.
The fatty acid amide hydrolase is responsible for further metabolizing p-aminophenol within the central nervous system to form N-acylphenolamine (AM404). AM404 interacts with several specific receptors within the midbrain and medulla, including transient receptor potential vanilloid 1 (TRPV1) and cannabinoid 1 (CB1). Since these receptors are co-localized modulators of pain perception and regulation, it is posited that acetaminophen induces analgesia by direct impacting the brain.\textsuperscript{37,38}

![Figure 1: Molecular structure of acetaminophen N-(4-hydroxyphenyl)acetamide](image)

Acetaminophen-derived nanoparticles might offer an advantage over other nanoparticles due to their origin from a well-known and widely accepted pharmaceutical compound. This could suggest a better biocompatibility and reduced side effects when introduced into the human body. It is hypothesized that if the acetaminophen-derived nanoparticles retain some properties or active components of the parent compound, there might be a dual benefit. Along with amyloid inhibition, they might provide symptomatic relief due to the analgesic properties of acetaminophen. This study seeks to explore the potential of these synthesized NPs not only as inhibitors of amyloid fibrils present in the body but also as vehicles for targeted drug delivery across the BBB, providing a dual approach for the treatment and management of NDs and type 2 diabetes.
Chapter 2: Development and Characterization of Nanoparticles using Acetaminophen as a Precursor.

ABSTRACT:

Nanoparticles (NPs) exhibit unique chemical versatility, and tailor-made biomedical applications. In this study, a hydrothermal synthesis approach was employed to produce nanoparticles derived from acetaminophen—a widely established analgesic and antipyretic compound. The synthesis processes were undertaken over time intervals of 4, 6, and 8 hours. Comprehensive characterization of the resultant NPs was achieved employing advanced techniques such as, Dynamic Light Scattering (DLS), Ultraviolet-Visible Spectroscopy (UV-VIS), Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR), $^1$H NMR spectroscopy. The overarching aim of these characterizations was to discern potential alterations in the physiochemical structure, which could be attributed to variations in the synthesis duration.

A salient component of this research centered on evaluating the NPs’ capability in preventing amyloidogenic pathways. For this purpose, a Thioflavin T (THT) assay was conducted to examine the presence of mature fibrils. Hen egg white lysozyme (HEWL) served as a model amyloidogenic protein, because of its well-characterized structure. This study offers a novel perspective on the biomedical applications of commonly used analgesics and sets the stage for further exploration of drug-derived nanoparticles in therapeutic avenues.
INTRODUCTION

In the realm of nanotechnology and neurodegenerative disease research, the utilization of nanoparticles emerges as a promising strategy to impede the aggregation of amyloid fibrils. Amyloid fibrils, composed of misfolded proteins, are implicated in the pathogenesis of several debilitating neurodegenerative disorders. By harnessing the unique physicochemical properties of nanoparticles, particularly their high surface area-to-volume ratio and tunable surface chemistry, researchers can engineer nanomaterials with specific functionalities. These nanoparticles can be tailored to interact with amyloidogenic proteins, thereby mitigating their propensity to form toxic fibrillary aggregates. Additionally, the unique structural attributes of NPs may enable them to bypass the BBB, a challenge that prevents the efficient delivery of therapeutic agents to the brain. This innovative approach not only offers a potential avenue for the development of novel therapeutic interventions but also exemplifies the synergy between nanoscience and biomedicine in addressing complex and pressing challenges in the field of neurodegenerative diseases.
Illustration 2: Schematic of the mechanism of ANPs inhibition of mature Aβ fibrils

Here, acetaminophen nanoparticles (ANPs) are assessed for their ability to interfere with the formation of amyloid fibrils. The Hen-Egg White Lysozyme (HEWL) was utilized as a prototypical amyloidogenic (fibril-forming) protein model. It's worth noting that the propensity of HEWL to form fibrils under certain conditions has been instrumental in studying lysozyme amyloidosis, a condition where lysozyme protein aggregates precipitate, resulting in multiorgan impairment.40 The process of fibrillogenesis, characterized by which a peptide (monomers) form insoluble aggregates of amyloid. This phenomenon is observed in prion-like proteins such as Aβ, tau, α-syn, and mHTT, all of which are crucial in the pathogenesis of neurodegenerative ailments and type 2 diabetes.2
**Thioflavin T as an Amyloid Dye**

Thioflavin T (ThT) is a benzothiazole dye with high aqueous solubility. It serves as a potent tool in both *in vivo* and *in vitro* studies for detecting and quantifying misfolded amyloid protein aggregates.\(^{41}\) Amyloid fibrils, implicated in neurodegenerative disorders like PD and AD, consist of insoluble aggregates of α-syn and Aβ with a predominant β-sheet structure.\(^{42}\) When bound to ThT, a dye with an affinity for β-sheet-rich formations, these fibrils become detectable.\(^{41,43}\)

ThT is predominantly used in the Thioflavin T assay, a technique that measures fluorescence intensity changes when the dye binds to amyloid fibrils. In its free state, ThT exhibits minimal fluorescence. Upon binding to amyloid fibrils, it produces a bright fluorescence signal accompanied by a shift in excitation wavelength from 385 nm to 450 nm and emission wavelength from 445 nm to 482 nm.\(^{44,45}\) This spectral shift confirms ThT's effective binding to amyloid aggregates and acts as a robust indicator of their presence and concentration in samples. The fluorescence triggered by binding offers a valuable perspective on the presence and abundance of these amyloid fibril structures. This study aims to determine whether the synthesized nanoparticles are capable of inhibiting fibril formation, potentially providing evidence of their efficacy as therapeutic approaches to neurodegenerative diseases.
MATERIALS AND METHODS

Chemicals and reagents

Acetaminophen was purchased from Wal-Mart Stores Inc. Hen Egg White Lysozyme (HEWL) was purchased from MP Biomedicals, LLC. Thioflavin T, Tris hydrochloride, and guanidinium hydrochloride were purchased from Fisher Scientific. Deuterium oxide (D$_2$O) was purchased from Sigma Aldrich USA. Acetonitrile-d$_3$ (CD$_3$CN) was purchased from Cambridge Isotope Laboratories, Inc. All chemicals utilized were of analytical grade and used without further purification. Milli-Q (MQ) water was used for all experimental procedures.

Synthesis of Acetaminophen Nanoparticles

Acetaminophen nanoparticles were synthesized using the one-step hydrothermal method. Acetaminophen (1000 mg) was dissolved in 40 mL MQ water. The solution was subjected to mixing using a magnetic stirrer in an Erlenmeyer flask for a duration of 10 min. Following this, the homogeneous solution was transferred to a hydrothermal autoclave reactor, which comprised an exterior stainless-steel jacket and an inner Teflon liner. The prepared samples were subjected to hydrothermal processing in a Isotemp general-purpose oven (Fisher brand) at 200°C for intervals of 4, 6, and 8 hours, respectively. After each heating period, the synthesized ANPs were left to cool to room temperature and then centrifuged at 5,000 rpm at a controlled temperature of 10°C for 10 minutes. The resulting mixture from each batch were filtered with a sterile syringe filter of pore size 0.22 μm and subsequently flash-frozen at -80°C for 24 hours. The purified samples were lyophilized and were preserved under vacuum conditions at room temperature until further analysis.
Illustration 3: Schematic representation of the preparation of Acetaminophen nanoparticles (ANPs).
Fibril inhibition ThT assay

Hen-Egg White Lysozyme (HEWL; MP Biomedicals) was used to prepare fibrils at concentration of 3 mg/ml (139 μM) in unfolding buffer containing a mixture of 2 M Guanidine Hydrochloride and 20 mM Tris-Hydrochloride (pH 7.5). ANP solutions were prepared at different concentrations and added to individual 5ml borosilicate glass tubes and placed in an incubator shaker (Multi-therm, Benchmark) at 60 °C, 550 rpm for 6 hrs. Post incubation, the samples exhibited turbidity, indicating potential protein aggregation. The fibril content in each sample was quantified utilizing an Olis DM45 Spectrofluorometer. ThT at a concentration of 20 μM was employed as the fluorescence intensity indicator. It was introduced into a quartz cuvette containing lysozyme, and the fluorescence intensity was recorded for a duration of 120 sec starting 10 secs after addition. The limits set were an excitation wavelength of 452 nm and an emission wavelength of 480 nm at a constant integration time of 0.1 s.

Dynamic Light Scattering (DLS) Measurements

Size distribution of the ANPs was evaluated by measuring the hydrodynamic diameter by dynamic light scattering (DLS) on a Zetasizer NanoZS (Malvern, UK). Acetaminophen-derived samples, including both raw (control) samples and those subjected to treatments of 4, 6, and 8 hours, were analyzed. The measurements consisted of 3 runs with a 60 sec duration for each, were performed at 25 °C. The viscosity (η) of 0.8872 mPa·s and a refractive index of 1.33 using Milli Q as the dilutant. The positional setting was for each measurement was fixed at 4.65 mm from the reference point.
1 mg of each sample was dispersed in 5mL of Milli-Q water. To ensure uniform dispersion and the breakdown of any aggregated particles within the sample, the solution underwent a sonication for a duration of 20 min prior to any further analysis. Following sonication, a 0.75 mL aliquot was extracted from each sample solution for particle size analysis. Measurements were conducted using disposable sizing cuvettes. Data was acquired and processed utilizing the Malvern Zetasizer NanoZS.

Physiochemical Characterization

The UV–vis absorption spectra of ANPs were measured between wavelength of range 200–600 nm. All observations were recorded using the Chemglass Life Sciences SpecMate spectrophotometer. For measurements of the fluorescence intensity DM45 Olis spectrofluorometer (Online Instrument System, Inc.) was used. Fourier – transform Infrared spectroscopy (FTIR) spectra were obtained on a Shimadzu IR PRESTINGE-21 instrument by applying the attenuated total reflection (ATR) technique.

NMR spectra were obtained on a Bruker-ADVANCE III 400 MHz spectrometer. $^1$H NMR spectra at 400 MHz had a TMS as the internal standard in deuterium oxide (D$_2$O) and acetonitrile-d$_3$ (CD$_3$CN) solvents. The multiplicities of the hydrogen nuclei energy absorption bands in the $^1$H NMR spectra are indicated according to the following: s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Chemical shifts (δ) are expressed in parts per million (ppm) using TMS (tetramethylsilane) as an internal standard and coupling constants (J) are expressed in Hz.
RESULTS AND DISCUSSION

**ThT assay**

The study delved into the impact of ANPs on mature HEWL fibrils, as delineated in Fig 2. This visual representation shows the detection of mature fibrils via ThT fluorescence across all samples. In Fig 2 A-D, the presence of only HEWL fibrils is detected by ThT as evidenced by a sharp increase in fluorescence intensity upon addition of ThT to the solution containing fibrils (red curve). Such enhancement can be rationally ascribed to the immediate binding of ThT to the mature fibrils. This offers insight into the affinity and kinetics of ThT towards the cross-β structures in the fibrils.

Further, the research ventured into discerning the dose-dependent ability of ANPs in preventing the transition of soluble monomeric HEWL to its fibrillar state. To achieve this, different concentrations of ANPs at 1mg/ml (black curve) and 5mg/ml (blue curve) were employed. Upon the addition of the varying concentrations to the solution, a visible decrease in the ThT fluorescence intensity relative to the untreated control and the acetaminophen raw material. The observed decline in fluorescence in both concentrations is indicative of the potential capability of ANPs to disintegrate or disaggregate mature HEWL fibrils.
Figure 2: Fibrillation inhibition Assay using HEWL. (a) ThT fluorescence of HEWL with acetaminophen raw material (b) ThT fluorescence of HEWL preincubation with 4hr-synthesized ANPs. (c) ThT fluorescence of HEWL preincubation with 6hr-synthesized ANPs. (d) ThT fluorescence of HEWL preincubation with 8hr-synthesized ANPs.
Dynamic Light Scattering

After evaluating the effectiveness of ANPs in inhibiting mature fibril formation using the ThT assay, Dynamic Light Scattering (DLS) was utilized to analyze the size distribution of ANPs and to detect any potential agglomerates and aggregates present in the samples. This technique measures the intensity of scattered light as a function of time and allows for the determination of the hydrodynamic diameter of particles in suspension. A comparison between the size distribution at different time intervals is shown in Fig. 3.

In Fig. 3: (a) The acetaminophen raw material exhibited an average hydrodynamic diameter of 378.9 nm and a polydispersity index (PDI) of 0.324. (b) The acetaminophen nanoparticles (ANPs) synthesized at 4 hrs displayed a size of 719.2 nm with a PDI of 0.297. (c) The 6 hr ANPs exhibited a diameter of 793.2 nm and a PDI of 0.370 and (d) ANPs synthesized at 8 hrs displayed a size of 823.8 nm and a PDI of 0.428. Results show there is a wide range of particle sizes. Typically, samples with particle sizes above 500 nm and PDIs above 0.5 are considered as “large and agglomerated.” However, such data not always warrant significant attention, due to the fact that the scattering of light is dominated by larger particles, even when a limited number of them are present. Therefore, this can overshadow the detection of “finer and smaller” particles, skewing the measurement in favor of a larger average particle size. In addition, this can increase the PDI values, which compromises the reliability of the results. Understanding the intricacies of particle surface modifications, especially when subjected to thermal variations, is imperative. Changes in surface conditions can influence the rate of diffusion and may also alter the perceived size of the particle. As stated previously, DLS measures the hydrodynamic diameter, and any ligands, ions, or molecules that are bound to its surface and travel with the particle in the
solvent. Such associated entities can cause the particle to present a larger effective diameter to DLS instruments compared to Transmission Electron Microscopy (TEM).

Figure 3: DLS analysis results for ANPs synthesized through various hydrothermal treatment intervals alongside raw acetaminophen material for comparison. (a) displays the raw acetaminophen (b) illustrates the ANPs subjected to a 4 hr hydrothermal synthesis process. (c) depicts the ANPs after a 6 hr hydrothermal treatment, and (d) shows the ANPs following an 8 hr hydrothermal synthesis.
UV-Vis absorption Spectroscopic Analysis

In Fig 4 shows the absorption spectra of the raw acetaminophen material depicted (black curve), alongside the absorption spectra for the acetaminophen nanoparticles (ANPs) synthesized over different reaction times of 4, 6, and 8 hours. The shift in absorbance observed at kmax 246 nm for Acetaminophen can be ascribed to the n–π* transition occurring in the C=O group at this wavelength. In contrast, the π-π* transition, which entails the excitation of an electron from a π bonding orbital to a π* anti-bonding orbital situated within the aromatic ring, typically takes place in the 290-300 nm range. The peak observed between 215 and 230 nm in the UV-Visible spectrum of Acetaminophen signifies the n-π* transition, particularly linked to the lone electron pair of the amide functional group within the compound. Changes in peak intensity can be ascribed to the influence of the solvent.

Figure 4: The absorption spectra of Acetaminophen solution at different reaction times.
ATR IR Spectroscopic Analysis of Raw Acetaminophen sample

ATR IR spectroscopic analysis is used to evaluate information about the functional group of the organic compounds by the stretching and bending vibrations of compounds in definite wavelength. The IR spectrum was recorded for 400 cm\(^{-1}\) to 3500 cm\(^{-1}\). In Fig 8, IR analysis revealed prominent peaks of the raw acetaminophen. The peaks are identified at 3322 cm\(^{-1}\), corresponding to N-H stretching in a secondary amide; at 3161 cm\(^{-1}\), associated with O-H stretching in a phenol group; 1651 cm\(^{-1}\), indicative of C = O stretching in an aromatic compound. Additional peaks are observed at 1610 cm\(^{-1}\) for C = C stretching (conjugated), 1562 cm\(^{-1}\) for N-H bending, 1506 cm\(^{-1}\) for asymmetrical C-H bending, 1435 cm\(^{-1}\) for O-H bending, 1371 cm\(^{-1}\) for C-H bending. Peaks at 1325 cm\(^{-1}\) &1226 cm\(^{-1}\) are attributed to C-N stretching.

Figure 5: ATR-IR spectra of raw acetaminophen material not subjected to hydrothermal synthesis.
<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>IR band assignment</th>
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<tbody>
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ATR IR Spectroscopic Analysis of ANPs 4 hrs

Fig 6 displays the ATR IR spectrum for the ANPs produced through a hydrothermal process at 200°C over a duration of 4 hrs. The IR spectral analysis revealed distinct peaks at various wavenumbers: 3022 cm\(^{-1}\) attributed to C-H stretching, 1653 cm\(^{-1}\) due to the stretching of C=O in an aromatic compound, 1613 cm\(^{-1}\) corresponding to conjugated C=C stretching, 1562 cm\(^{-1}\) associated with N-H bending, 1506 cm\(^{-1}\) for asymmetrical C-H bending, 1452 cm\(^{-1}\) indicative of O-H bending, 1374 cm\(^{-1}\) for C-H bending, and 1213 cm\(^{-1}\) for C-N stretch. Notably, there were no prominent peaks observed for N-H and O-H stretching in the spectrum, which is a deviation from the Raw Acetaminophen sample spectrum thus highlighting changes in the structural composition.

Figure 6: ATR IR spectrum of Acetaminophen dissolved in Milli-Q water, post-hydrothermal synthesis at 200°C for 4 hrs.
<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>IR band assignment</th>
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<td>659.55</td>
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</table>
ATR IR Spectroscopic Analysis of ANPs 6hrs

**Fig 7** displays the ATR IR spectrum of ANPs synthesized via a hydrothermal method at 200°C for a period of 6 hours. The IR spectral analysis reveals prominent peaks at the following positions and corresponding bond vibrations: 3322 cm\(^{-1}\) indicating N-H stretching of a secondary amide, 3108 cm\(^{-1}\) for O-H phenol stretching, 1653 cm\(^{-1}\) due to C=O stretching in an aromatic structure, 1610 cm\(^{-1}\) associated with conjugated C=C stretching, 1562 cm\(^{-1}\) for N-H bending, 1503 cm\(^{-1}\) for asymmetrical C-H bending, 1435 cm\(^{-1}\) for O-H bending, 1369 cm\(^{-1}\) corresponding to C-H bending, and lastly, 1226 cm\(^{-1}\) for N-H stretch.

![ATR IR spectrum of ANPs](image)

**Figure 7**: ATR IR spectrum of Acetaminophen dissolved in Milli-Q water, post hydrothermal synthesis at 200°C for 8 hrs.
<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>IR band assignment</th>
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<tr>
<td>3322.15</td>
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<td>659.55</td>
<td>Ar-C-H bending</td>
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</tbody>
</table>
ATR IR Spectroscopic Analysis of ANPs 8 hrs

Fig 8 presents the ATR IR spectrum of ANPs that were produced using a hydrothermal process at 200°C for 8 hours. The IR spectral analysis reveals prominent peaks, these include: 3324 cm\(^{-1}\) for N-H stretching for secondary amide, 3111 cm\(^{-1}\) for O-H phenol stretching, 1653 cm\(^{-1}\) attributed to C = O stretching in an aromatic compound, 1610 cm\(^{-1}\) for C = C stretching (conjugated), 1562 cm\(^{-1}\) due to N-H bending, 1506 cm\(^{-1}\) for asymmetrical C-H bending, 1437 cm\(^{-1}\) associated with O-H bending, 1371 cm\(^{-1}\) for C-H bending, and finally 1229 cm\(^{-1}\) for N-H stretching.

Figure 8: ATR IR spectrum of Acetaminophen dissolved in Milli-Q water, post-hydrothermal synthesis at 200°C for 8 hours.
<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
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<td>659.55</td>
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</table>
$^1$H NMR of Acetaminophen Raw material (a)

White solid; $^1$H NMR (CD$_3$CN, 400 MHz), $\delta$ 7.36 (d, $J = 8.9$Hz, 2H), 6.76 (d, $J = 8.9$Hz, 2H), 2.04 (s, 3H)

(a)

Figure 9: $^1$H NMR (CD$_3$CN) Acetaminophen raw material
$^1$H NMR Acetaminophen sample 4 hrs (b)

Redish solid; $^1$H NMR (D$_2$O, 400 MHz), δ 7.24 (d, J = 9.0Hz, 2H), 7.16 (d, J = 9.0Hz, 2H), 6.94 (d, J = 8.96Hz, 2H), 6.90 (d, J = 8.96Hz, 2H), 6.81 (s, 1H), 2.14 (s, 3H), 1.96 (s, 3H).

(b)

Figure 10: $^1$H NMR (D$_2$O) Acetaminophen sample at 4 hrs.
\(^1\)H NMR Acetaminophen sample 6 hrs (c)

Redish solid; \(^1\)H NMR (D\(_2\)O, 400 MHz), \(\delta\) 7.24 (d, \(J = 9.0\)Hz, 2H), 7.16 (d, \(J = 9.0\)Hz, 2H), 6.94 (d, \(J = 8.96\)Hz, 2H), 6.90 (d, \(J = 8.96\)Hz, 2H), 6.81 (s, 1H), 2.14 (s, 3H), 1.96 (s, 3H).

Figure 11: \(^1\)H NMR (D\(_2\)O) Acetaminophen sample at 6 hrs.
$^1$H NMR Acetaminophen sample 8hrs (d)

Redish solid; $^1$H NMR (D$_2$O, 400 MHz), $\delta$ 7.10 (d, $J = 8.96$Hz, 2H), 6.76 (d, $J = 8.96$Hz, 2H), 2.00 (s, 3H)

Figure 12: $^1$H NMR (D$_2$O) Acetaminophen sample at 8 hrs.
Chapter 3: Conclusion

By and large, the hydrothermal method employed in this research proved to be highly effective for the synthesis of acetaminophen nanoparticles (ANPs), maintaining a stable temperature throughout various durations, and successfully resulting in their formation. The ThT assay results were pivotal, showcasing the ANPs' ability to inhibit the formation of amyloid fibrils, thereby establishing their promising role as a potential therapeutic solution for neurodegenerative diseases and type 2 diabetes. This marks a remarkable progression from the initial state of raw acetaminophen, which exhibited no inhibitory impact on fibril formation.

The results presented in this thesis has shed light on the crucial influence of thermal exposure duration in defining the ANPs’ characteristics. A clear relationship has been established between the duration of heat application and physiochemical properties, such as nanoparticle size, absorbance capabilities, and functional groups. The DLS results revealed an apparent increase in particle size due to prolonged thermal exposure, leading to a greater degree of agglomeration. The UV-Vis spectroscopy highlighted a significant shift in absorbance from the raw material in comparison to all ANPs, indicating substantial changes in their properties. Contrastingly, ATR-IR spectroscopy analyses highlighted similarities in certain functional groups across all samples, maintaining a level of consistency. Nonetheless, ¹H NMR was utilized to confirm the structural alterations in the ANPs and to detect other potential compounds. This technique revealed that prolonged heating resulted in the emergence of smaller, distinct peaks, possibly signaling molecular-level vibrations. Despite these differences, the observed consistency in peaks within both aromatic and aliphatic regions throughout all samples reinforced the similarity of the ANPs to the raw
material. Ultimately, this research lays down a solid groundwork, elucidating how thermal processes can be precisely controlled to customize material properties for specific applications, thereby fostering innovation and progress in the field of biotechnology.
References


44. Hellstrand, E., Marginean, D. & Larsson, J. Fluorescent molecules as probes for characterization of amyloid β fibrils.
Vita

Hannia Mendoza-Dickey graduated with a Bachelor of Science (BSc.) degree in Biology from the University of Texas at El Paso (UTEP) in 2022. She initiated her research endeavors during her master’s studies and currently serves as a teaching assistant in the Department of Chemistry and Biochemistry at UTEP. Under the mentorship of Dr. Hildenbrand her initial research focused on utilizing recombinant DNA techniques to induce the expression of the Cannabichromenic acid (CBCA) synthase enzyme in *Pichia pastoris* yeast. This innovative expression system has been intricately designed to produce substantial quantities of CBCA synthase, aiming to synthesize CBCA for its potential use as a pharmaceutical intervention in treating neurodegenerative conditions and depression. Hannia’s profound interest and passion for cannabis research has culminated in her authoring several articles for Grow Magazine and Cannabis Science and Technology. Her current research revolves around the synthesis and testing of acetaminophen derived nanoparticles that can prevent the trajectory leading from the soluble-to-toxic form of amyloid-forming proteins with Dr. Narayan. The emphasis of her research resides in exploiting chemical frameworks that can eventually cross the Blood Brain Barrier and prevent xenotoxicant associated neuronal injury and demise which are casuals for neurodegeneration. Hannia is a tenacious and hardworking individual. She is committed to persevering in her work and aims to contribute to the scientific community through publishing research focused on uncovering treatments for debilitating conditions like Alzheimer's and Parkinson's disease which impact countless individuals both within the United States and around the world.